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Evaluation of Selected Subacute Effects of the Nitrotoluene Group of Munitions
Compounds on Fish and Potential Use in Aquatic Toxicity Evaluations

Final Report

William Ralph Hartley

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Juvenile bluegills were exposed to 0.05 mg/L, 0.50 mg/L, 1.0 mg/L, 2.0 mg/L, 4.0 mg/L, 5.0 mg/L and 8.0 mg/L 2,4-DNT for eight weeks. Both first and second order growth constants indicated reduced growth rates with increasing 2,4-DNT concentration. The threshold concentration for significant growth rate reduction was 0.05 mg/L 2,4-DNT.

No histological abnormalities were found in the digestive tract, pancreas, integument, heart, gonad, head kidney and spinal cord. Significant

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histopathological responses were observed in liver, spleen, trunk kidney, lateral line and gill in fish exposed to 0.5 mg/L - 8.0 mg/L 2,4-DNT for 45 - 56 days.

The 2,4-DNT was rapidly absorbed (24-96 hours), reached relatively low bio-concentration levels and was rapidly eliminated (24-72 hours) when fish were placed in a 2,4-DNT free environment.

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EVALUATION OF SELECTED SUBACUTE EFFECTS OF

2,4-DINITROTOLUENE ON THE BLUEGILL SUNFISH

LEPOMIS MACROCHIRUS

DISSERTATION OF A STUDY UNDERTAKEN
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS
FOR
THE DEGREE OF

DOCTOR OF SCIENCE
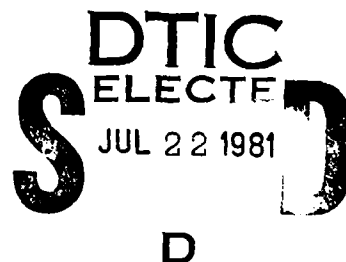
BY

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March 4, 1981

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ABSTRACT

This study was undertaken to establish baseline histo-
logical and growth data on the juvenile bluegill sunfish,
Lepomis macrochirus, under laboratory conditions. By means
of 8-week exposures, the sublethal histological and growth
responses of the bluegill sunfish to subacute concentrations
of the munitions toxicant, 2,4-dinitrotoluene (2,4-DNT) were
evaluated. Uptake (two week), distribution and elimination
(one week) of C^{14} (ring labeled) 2,4-DNT were evaluated to
determine target organ systems, routes of entry and biocon-
centration.

Normal growth in juvenile bluegills was described and
four (zero, first, and second order and LOG-LOG) models were
tested for application. Bluegill growth was adequately de-
scribed by the first and second order models. First order
growth constants for juvenile bluegills at 21°C were 0.0132-
0.0144 day^{-1}.

Juvenile bluegills were exposed to 0.05 mg/l, 0.50
mg/l, 1.0 mg/l, 2.0 mg/l, 4.0 mg/l, 5.0 mg/l and 8.0 mg/l
2,4-DNT for eight weeks. Both first and second order growth
constants indicated reduced growth rates with increasing
2,4-DNT concentration. The first order growth constants were
significant at all concentrations of 2,4-DNT tested when ex-
posed groups were compared to controls and to each other
(α=0.01). The threshold 2,4-DNT concentration for significant
growth rate reduction was 0.05 mg/l 2,4-DNT. The second order
model applied equally well and has not been previously used
as a model for juvenile fish growth under toxic conditions.

In comparison with other teleost fishes, the juvenile
bluegill histology was similar but there were unique varia-
tions particularly in the stomach portion of the gut. However,
tissue structures, in general, were similar to the extent that
form and function appeared similar to other teleost.

No histological abnormalities were found in the diges-
tive tract (gut), pancreas, integument, heart, gonad, head
kidney and spinal cord. Significant histopathological re-
sponses were observed in liver, spleen, trunk kidney, lateral
line and gill in fish exposed to 0.5 mg/l-8.0 mg/l 2,4-DNT
for 45 to 56 days. These lesions included hypertrophy of
gill lamellae, hepatic lipid accumulation with associated
nicrotic foci, atypical trunk kidney tubules with associated
tubule necrosis, and atypical neuromast cells with necrotic
epithelium in the lateral line mechanoreceptors. The uptake
distribution and elimination of 2,4-DNT in bluegills indicated
that target organs were the brain, kidney, liver, stomach/
intestine and gills. Major routes of entry based on uptake

rates were the gill and stomach/intestine.

The 2,4-DNT was rapidly absorbed (24-96 hours), reached relatively low bioconcentration levels and was rapidly eliminated (24-72 hours) when fish were placed in a 2,4-DNT free environment.

CHAPTER I

INTRODUCTION AND OBJECTIVES

INTRODUCTION AND OBJECTIVES

INTRODUCTION

Biomonitoring of military and industrial discharges is developing as a useful means of protecting aquatic ecosystems. Due to the complexity of most discharges, the use of chemical analysis as a means of discharge monitoring does not adequately evaluate the impact on the organisms in the receiving waters. Acute toxicity bioassays using indigenous species as bioassay organisms are already required by some National Pollutant Discharge Elimination System (NPDES) permits at military and industrial installations. This research *evaluates selected sublethal responses of the bluegill sunfish, Lepomis macrochirus*, to the munition compound, 2,4-dinitrotoluene (2,4-DNT) as a model for subacute response.

Current toxicological research on the effect of munitions compounds are directed toward establishing safe concentrations for use as discharge standards in government and industry. Establishing incipient lethal levels is currently being accomplished by conducting acute toxicity bioassays. Early studies by Bliss (1937, 1940), Doudoroff (1952), and Weiss and Botts (1957) indicate that acute toxicity studies provide an estimation of the relative toxicity and provide basic information about a compound. Acute toxicity data also provides inferential information (Sprague 1969, 1970, 1971) on such variables as organism susceptibility, environmental factors and rate of chemical deactivation.

Recognizing the need for information on the biological effects of low level exposure to environmental toxicants, long-term chronic toxicity studies have been developed that evaluate the effect of the toxicants on the reproduction, survival and growth of species through all stages of the life cycle. Studies by Mount and Stephan (1967, 1969) and Pickering (1974) established the need for toxicant evaluation via chronic exposure throughout the life cycle of the fish. Chronic studies are currently used with acute toxicity data to establish discharge standards (both pipe and in-stream) to protect the aquatic environment.

There is a hiatus in the development of aquatic toxicology that does not exist in mammalian toxicology. The hiatus is the paucity of research on short-term subacute histological responses of fish to sublethal concentrations of toxicants. The measurement of specific histological changes in the fish exposed for short periods to sublethal concentrations of environmental toxicants may provide a sensitive method for predicting the effects of chronic exposure on survival. Such histological responses may also be useful for developing more effective biomonitoring programs. Bioassays using histological responses to evaluate microchemical environmental contaminants were first extensively reviewed by Warner (1964, 1967). Subsequent studies by Gardner and Yevich (1970), Jackim et al. (1970), Life (1970), Bell (1968), McKim et al. (1970), Couch et al. (1979) and others have demonstrated that significant histological and physiological changes in organisms from non-lethal levels of organic and inorganic toxicants

occur. As currently practiced in mammalian toxicology, sub-acute responses in fish will assume a more important role in aquatic toxicology evaluations and be a means of providing a more efficient way of evaluating chronic toxicity. Subacute responses may also be used in providing an early warning system in the biomonitoring of discharges. Since most discharge standards for toxicants in NPDES permits are well under the acutely toxic concentrations, acute toxicity bioassays in biomonitoring programs are of limited usefulness in preventing severe violations.

This research describes and evaluates the subacute effects (histological and physiological) produced in the bluegill sunfish, Lepomis macrochirus, when exposed to sublethal concentrations of 2,4-dinitrotoluene for eight week periods.

OBJECTIVES

1. Establish baseline histological and physiological data on the bluegill sunfish under normal laboratory conditions.

2. Conduct 8-week exposures of the bluegill sunfish to sublethal concentrations of 2,4-DNT under flow-through bioassay conditions.

3. Describe all sublethal histological effects and selected physiological responses resulting in the bluegill sunfish due to 2,4-DNT exposure.

4. Predict the possible uses of short-term responses (8-weeks or less) as a means of evaluating toxic effect in the environment and discuss potential use in biomonitoring systems.

CHAPTER II

LITERATURE REVIEW

LITERATURE REVIEW

Bioassay Organism

The desirability of having a standard fish species as
a bioassay organism for reproducibility of test results has
been proposed and evaluated by Marking (1966), Lennon (1967),
Cairns (1969) and Sprague (1970). Most of those researchers
agree that a standard fish species for freshwater toxicology
evaluations would be of value. Adelman and Smith (1976) re-
viewed the more important criteria for selection of any strain
as a standard fish for bioassay work. The bluegill sunfish
(Lepomis macrochirus) meets these commonly accepted standards.
They have frequently been used as bioassay organisms in acute
toxicity studies and have demonstrated a constant response to
a broad range of toxicants when tested under similar environ-
mental conditions (Cairns, 1957; Ball, 1967; Coleman, 1974;
Mauck and Olsen, 1976; Cardwell et al., 1976). The fish are
available in large quantities with adequate control of quality
and environmental history. They are easily transported and
available in any desired size (Brauhn and Schottger, 1975).

Sources of 2,4-Dinitrotoluene (DNT)

The evolution of 2,4-DNT as an environmental problem
is associated with 2,4,6-trinitrotoluene (Alpha TNT, 2,4,6-
TNT, TNT, trotyl, triton, tritol, or trilite) production and
use. The most important of the six isomeric trinitrotoluenes
is 2,4,6-TNT. It was first prepared by Wilbrand in 1863.
The byproduct, 2,4-DNT was described by Demselben (1882).
Trinitrotoluene was produced on an industrial scale in the

German Republic in 1891. In 1901, Germany started to produce
2,4,6-TNT on a commercial scale. In 1902, the German Army
adopted it as a basic filling for shells. Following the Ger-
man example, nearly all countries used 2,4,6-TNT by the out-
break of World War II. The use of 2,4,6-TNT during World War
I was limited by the available supply of toluene by the coke
industry. It was the industrial development of synthetic
toluene from petroleum just prior to World War II that made
available to the United States an almost unlimited supply of
toluene to produce 2,4,6-TNT (Military Explosives, 1967).

Trinitrotoluene can be manufactured by one, two or
three-stage nitration processes, with toluene and mixed acid
as the raw materials. Maximum yield, purity of product,
greater control of acid concentration, and temperature control
are the advantages of the three stage process. During World
War II, the 'direct nitration process' was developed in which
toluene, mononitrotoluene and dinitrotoluene were added to the
mixed acid. The nitration cycle was reduced from 2 hours to
45 minutes due to improvements in the process. This allowed
a potential production of 3,600,000,000 pounds of TNT per year
(Military Explosives, 1967).

When toluene is nitrated in the mono-nitrostage, the
chief products are:

| toluene | ortho-
nitrotoluene
(62%)* | para-
nitrotoluene
(33.5%)* | meta-
nitrotoluene
(4.5%)* |
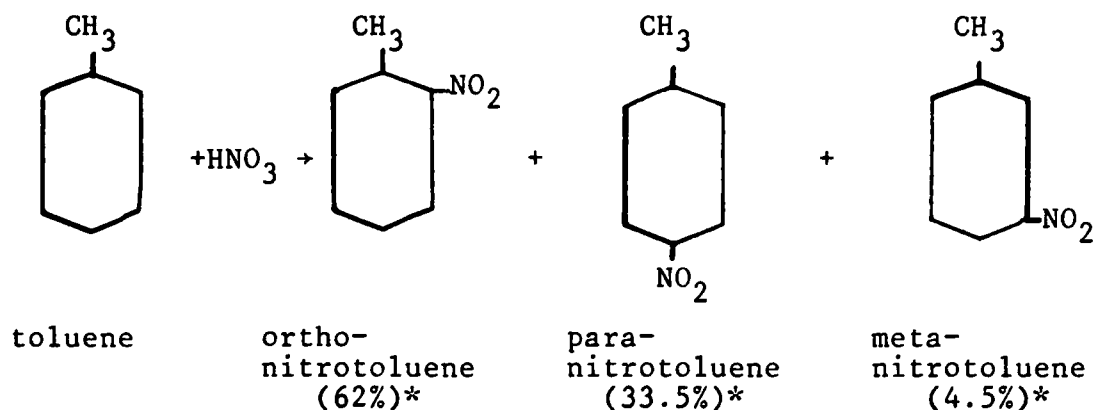
*approximate percentage of each isomer as a result of
mononitration of toluene

Since these compounds have melting points of 10.6°, 51.3°,
$15.5^\circ C$, respectively, the product is an oily liquid at ordi-
nary room temperature. When this is subjected to dinitration,
the products are the following dinitrotoluenes:

| 2,4-DNT
(74.8%)* | 2,6-DNT
(20.7%)* | 3,4-DNT
(2.5%)* | 2,3-DNT
(1.1%)* | 3,6-DNT
(0.9%)* |
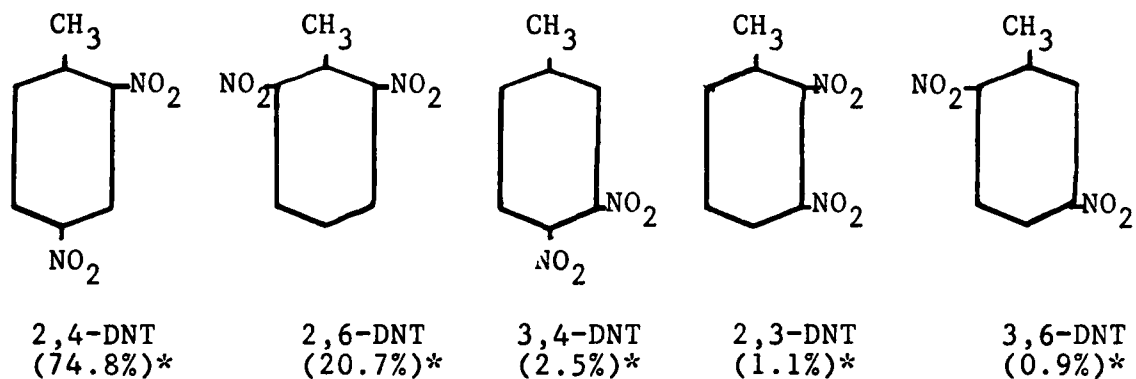
*approximate percentage of each isomer as a result of
dinitration of toluene

The mixture of these products is an oily liquid at temperatures
which allow separation from spent acid and subjection to tri-
nitration. The resulting mixture consists of approximately
95% 2,4,6-TNT. Impurities at this final stage include other
TNT isomers and some DNT isomers (Military Explosives, 1967).

Trinitrotoluene is produced in either a batch or continuous mode. In the batch process, the dinitrotoluenes flow to a final reactor in which most of the TNT formation occurs. A small amount of 2,4-DNT in this transfer step remains in the process pipeline and drips into the cooling water discharge system. The cooling water flows to surface streams with no further treatment. In the continuous process, the toluene flows into and proceeds through a series of nitrators. The aqueous acid and organic phases are separated between the nitrators. Dinitrotoluene may be lost due to leaks or discarded process control/assay samples (Small and Roxenblatt, 1974).

In the batch process, 2,4,6-TNT is crystalized, washed with sodium carbonate to remove residual acid and washed with 16% sodium sulfite solution to remove TNT isomers other than 2,4,6-TNT. After each washing, these waters are discharged. Collectively, this "charge" water is called "red water". In the continuous process, the crude liquid TNT is washed with water to remove acids. The molten 2,4,6-TNT is washed with sodium sulfite solution. The water from the first wash is called "yellow water". The yellow water is recycled to the nitration process or incinerated. The second wash with sodium sulfite is also called red water, but it is not the same composition as the red water from the batch process. However, red water from either process has considerable amounts of unreacted 2,4-DNT (Small and Rosenblatt, 1974).

Red water is discharged, incinerated or sold to paper mills for its sulfur content. Since red water sold to paper mills must be concentrated to 30% solids, 2,4-DNT is discharged

into the atmosphere when concentration occurs in open vats or lagoons. Some 2,4-DNT is found in discharge from scrubber systems. The spent acids from the nitration process are processed to recover nitric and sulfric acid. The wastewater from this treatment process is discharged to surface streams. Some 2,4-DNT also occurs in those discharges (Small and Rosenblatt, 1974).

The military requirement for 2,4-DNT specifies a minimum melting point of 65.5°C, which corresponds to 92% purity. Although 2,4-DNT can be prepared from toluene, the current approach is to purchase commercial 2,4-DNT. It is then purified by a process known as "sweating". The commercial material is approximately 75% 2,4-DNT and 20% 2,6-DNT. The commercial product is melted and subjected to a controlled cooling-heating program. In the cooling step, 2,4-DNT crystallizes, while the liquid impurity-rich fraction is drained off (or 'sweated'). This fraction includes most of the 2,6-DNT (57%) and 43% 2,4-DNT. The remaining solid (65% of the initial amount) is removed for use. The impurity rich fraction is added to the dinitrification stage product of the previously described TNT production process. The 2,4-DNT produced by "sweating" is used in smokeless propellant powder. The propellant is 85% nitrocellulose, 9% 2,4-DNT and trace amounts of diphenylamine and dibutylphthalate. The main function of 2,4-DNT in smokeless propellant powder is to control the burning rate of the propellant (Small and Rosenblatt, 1974).

The "sweating" process generates only minor amounts of 2,4-DNT wastewater. The most significant amounts of 2,4-DNT

containing wastewaters arise from the production of smokeless powder and 2,4,6-TNT (Small and Rosenblatt, 1974). Jurinski et al. (1975) estimate that the daily volume of water from a single plant may be as high as one-half million gallons. They estimate the total solubility of nitrated constituents can range from 80 to 100 mg/l.

Smokeless powder production begins with the removal of water from wrung cellulose. The nitrocellulose is next pressed into circular blocks at low pressure. Then, ethanol (95% volume/volume) is added to the blocks as the blocks are squeezed at increasing pressures. This operation, called the "dehy operation", prevents residual water from interfering with subsequent steps. Each block processed contains 38 lb. nitrocellulose, 9 lb. ethanol and 1 lb. water. The blocks are broken into small lumps and added to an 83.6% ether solution to which 2,4-DNT, dibutylphthalate and diphenylamine have been previously added. About 25.5 lb. of solution is used per block processed. The nitrocellulose forms a colloidal dispersion with the alcohol-ether-additive mixture. The colloidal mass, a dough consistency, is first pressed into blocks and then into "macaroni" shaped strips with high pressure to speed up the dispersal process. The strips are screened, drawn through dies and cut to desired length to form grains. These grains are usually cylindrical and sometimes serrated around the cylinder walls. They also usually have multiple perforations in the axial direction. These intricate shapings promote a uniform propellant burning rate. The grains are

shrunk to final size by drawing off solvent in warm oxygen-
free gas (<u>solvent recovery process</u>), submerged in water
maintained at 165°F to remove residual solvent (<u>water dry
process</u>), and dried in air (Small and Rosenblatt, 1974).

The major constituent of water discharged in the sol-
vent recovery and water dry process is 2,4-DNT. Propellant
grains subjected to the solvent recovery process are washed
from the tanks and covered with water during transport to
the water dry process. This water is discharged without
treatment. The process water used in the water dry process
is also discharged without treatment. There is some 2,4-DNT
in wastewater from various screening and cutting operations.
However, the amounts of water involved are smaller. Some
2,4-DNT occurs in wastewaters associated with floor washdowns
from process buildings and 2,4-DNT screen house where bulk
2,4-DNT is screened to proper size prior to being mixed with
ether. The volumes of water involved are not large compared
with the discharges from the solvent recovery and dry pro-
cesses (Small and Rosenblatt, 1974).

Ball powder is a double-base propellant. The nitro-
cellulose needed for ball powder is reclaimed from stores of
smokeless powder that are no longer satisfactory for military
use. The obsolete smokeless powder is washed into a extract
tank. It is leached with a solution of 88 percent benzene
and 12 percent ethyl acetate. The leacheate solution contains
2,4-DNT, dibutylphthalate and diphenylamine and is sparged
to recover benzene and acetate. The 2,4-DNT is introduced

to the wastewater when the water used to transport smokeless powder to the tank comes into contact with the residual solution in the bottom of the tank. The wastewater is then discharged without treatment to the sewer system (Small and Rosenblatt, 1974).

Spanggord et al. (1978) did an exhaustive study of the chemical composition of wastewater generated during the purification and production of 2,4,6-TNT at Volunteer Army Ammunition Plant in Chattanooga, Tennessee. All wastewaters at this plant were concentrated by evaporation. Called "condensate water", the identification of components, and development of a representative ratio of discharged components based on sampling studies over a 12-month period were accomplished. Only major components were identified and quantified in the chromatographic profile. As the identification of major components were confirmed (through laboratory synthesis of authentic compounds), quantitative data for these components were also generated. Cluster analysis of the data using a computer program developed by Sanford Research Institute (SRI) International resulted in the relative percentages of condensate components along with concentration. The results are shown in Table 1. As can be observed, 2,4-DNT is the major chemical component in terms of relative percent (44.2-52.9 percent) and concentration (5.3-13.7 mg/1).

Upon analysis of a larger data base, Spanggord et al. (1978) determined the relative distribution of components to be expected in a condensate discharge at Volunteer Army Ammunition plant. This analysis correlated well with the results

Table 1.

RELATIVE PERCENTAGES OF CONDENSATE COMPONENTS

DERIVED FROM COMPUTER CLUSTERS

(Spanggord et al., 1978)

Condensate Component	Cluster #1		Cluster #2		
	mg/l	%	mg/l	%	Av %
1,3-Dinitrobenzene	1.09	9.1	2.9	11.2	10.2
2,6-Dinitrotoluene	2.66	22.1	5.98	23.9	22.7
2,5-Dinitrotoluene	0.10	1.0	0.3	1.0	1.0
2,4-Dinitrotoluene	5.3	44.2	13.7	52.9	48.6
3,4-Dinitrotoluene	0.2	1.7	0.54	2.1	1.9
2,3-Dinitrotoluene	0.4	3.3	0.53	2.0	2.7
2,4,6-Trinitrotoluene	0.5	4.2	0.54	2.1	3.2
4-Amino-2,6-DNT	0.34	2.8	0.26	1.0	1.9
N-Nitrosomorpholine	0.10	1.0	0.13	0.5	0.7
N-Morpholinoacetonitrile	0.24	2.0	0.24	1.0	1.5
3-Amino-2,4-dinitrotoluene	0.78	6.5	0.31	1.2	3.9
3-Amino-2,6-dinitrotoluene	0.14	1.2	0.27	1.0	1.1
5-Amino-2,4-dinitrotoluene	0.15	1.3	0.18	0.7	1.0

of cluster analysis (see Table 1). The 90th percentile concentration and relative concentration (percent) for 2,4-DNT were 14.7 mg/l and 43.377 percent, respectively.

Information on measured and actual wastewater volumes and resultant concentrations of 2,4-DNT in the environment is limited. Table 2 is a summary of a few discharges that characterize the environmental problem and pollutional load (Small

Rosenblatt, 1974). Based on the flows cited and a 40 lb/day
loading of 2,4-DNT, the concentration in the receiving stream
was 0.01-0.015 mg/l 2,4-DNT.

Table 2.

TYPICAL 2,4-DNT DISCHARGES FROM RADFORD
ARMY AMMUNITION PLANT (Small and Rosenblatt, 1974)

Flow	Source	2,4-DNT mg/l	Mass Loading
5.0 MGD	TNT Prod., Scrubber Cooling/Water	1.4 mg/l	1.4 lb/day
1.08 MGD	Smokeless Powder Production	4.0 mg/l	1 lb/day
22,000 gal/day	Water Dry Process	29-210 mg/l	37 lb/day

Analytical Chemistry of 2,4-Dinitrotoluene

In the manufacture of 2,4,6-TNT, analytical methods
have been required to monitor the individual unsymmetrical TNT
isomers in symmetrical 2,4,6-TNT. An early method developed
by Halfter (1947) was based upon the selective reaction be-
tween sulfite ion and one of the nitrogroups of an unsymmetri-
cal TNT molecule. This method determined the total amount of
unsymmetrical TNT but failed to distinguish between the indi-
vidual unsymmetrical isomers. Subsequently, methods utilizing
infrared spectrometry (Pristera, 1953; Pristera et al., 1960),
thin layer chromatography (Yasvda, 1964) and paper chromatog-
raphy were reported. However, these methods generally lacked
sensitivity, and/or qualitative accuracy at low concentrations.

Gehring and Shirk (1967) were able to separate and
identify the isomers of DNT and TNT by gas chromatography.
They identified the isomers found in crude and refined TNT
production by comparing retention times of peaks to those for
pure samples of each isomer. Their system separated individ-
ual DNT and TNT isomers except for the 2,3,6-TNT and 2,4,6-
TNT pair. Three of the DNT isomers were poorly resolved.
The study indicated that gas chromatography techniques could
be applied to analyzing crude and refined TNT for TNT and DNT
isomers of 0.02 to approximately 3.0 percent. This method
required the availability of high-purity isomers for the
preparation of external standards and frequent checks on in-
strument calibration. The procedure also did not indicate if
isomers of mononitrotoluene and other nitration products could
be determined. Dalton et al., (1970) developed a method that
permits determination of toluene, trinitrobenzene, and mono-,
di-, and tri-nitrotoluenes. Three of the DNT isomers were
poorly resolved. Their method required a determination of
flame ionization response factors, eliminated preparation of
external standards and repeated instrument calibration. This
permitted more rapid in-process analysis of TNT production
samples. The column used for separation was 10% uc-w 98 sili-
cone rubber on 80-100 diatoport-S detector. Detector response
factors were determined and used to calculate relative percent
components and eliminated time consuming calibration and
achieved better reproducibility.

Gas chromatographic methods to separate all six DNT
isomers (Parsons et al., 1961) using a hot wire detector

resulted in separation adequate for nitration studies. Liquid chromatographic (Walsh et al., (1973) and high speed liquid chromatographic (Duali and Juhasz, 1974) methods have been reported for 2,4-DNT.

Jurinski et al., (1975) made a comparison between a gas chromatographic analysis method and an automated colorimetric method for 2,4,6-TNT in wastewater. The gas chromatographic method was far more useful in situations which required quantitative information about some isomers of di- and trinitrotoluene. The automated method required less work per analysis and gave a positive response for all trinitrotoluene compounds, including some degradation compounds. These factors made the automated method desirable for a routine surveillance program while the gas chromatographic method was better for exact isomer analysis. Pella (1976) constructed a vapor generator to produce known vapor concentrations of explosives such as 2,4,6-TNT, 2,4-DNT, 2,6-DNT and ethylene glycol dinitrate below 1 part per billion (ppb) by volume for calibrating trace explosives vapor detectors. The system was temperature controlled which permitted a wide range of equilibrium vapor concentrations to be generated. These vapor concentrations were diluted by single-stage dynamic gas blending to obtain concentrations as low as 0.05 ppb. A quantitative gas chromatograph procedure was developed to evaluate the system by measuring output vapor concentrations. The system error was usually within 15 to 20 percent of the expected values. The applicability of the system for calibration purposes was demonstrated by performance data obtained with

the commercial trace explosive vapor detectors.

Treatment of TNT Wastewater

The cleaning of the intensely red colored wastewater from the production of TNT has been a problem. These wastewaters contain 2,4,6-TNT, unsymmetrical trinitrotoluenes, dinitrotoluenes and chemically converted isomers in the form of sodium salts of trinitrotoluenes, dinitrotoluenes and chemically converted isomers in the form of sodium salts of trinitrotoluene sulfonic acids. Some 2,4,6-TNT is produced by nitration of toluene in steps, and both the mononitro phase and the dinitro phase are subjected to washing with alkali and extensive use of wastewater. The problem of the wastewater from a nitration operation has been evaluated in the United States and other countries. Ruchhoft et al., (1945) reported that conventional sewage treatment practices are not effective on TNT containing wastewater. Biological degradation was reduced and 1.0 mg/l 2,4,6-TNT retarded biological activity. Settling, filtration and granular activated carbon adsorption (sorption) is an effective treatment sequence being practiced in the United States and Switzerland (Angst and Brom, 1967).

Trinitrotoluene wastes (including 2,4-DNT) originate in explosive manufacturing plants, shell and bomb reclamation plants. The latter operation is often termed "depot". Here the water is recycled without significant treatment until shut-down. These two categories of plants have used activated carbon. In general, there has been a high interest in granular activated carbon for treatment of these wastes.

In TNT manufacture, the desired product is 2,4,6-TNT. Isomers of 2,4,6-TNT and 2,4-DNT are washed from the product via sulfite treatment. The waste from TNT manufacture thus contains acid reactant, red water from sulfite treatment and cooling water (Schott et al., 1943). In addition to the above 2,4,6-TNT is one of the many contaminants found in TNT manufacturing wastes and would be the principal constituents in shell loading plant wastes. The high relative proportion of 2,4-DNT in these wastes has been previously discussed. Both types of facilities probably manufacture and/or handle explosion indicators (lead azide) and boosters (tetrylnitrated dimethylaniline) and these could be present in the wastewater. Activated carbon plants are being used in Burlington, Iowa and Dottikon, Switzerland for the treatment of TNT manufacturing wastes. At Burlington, the treatment sequence is as follows: settling, diatomaceous earth filtration; and granular activated carbon treatment (Angst and Brom, 1967; Rosenblatt et al., 1971).

Human Exposures to 2,4-Dinitrotoluene

Human poisoning with dinitrotoluene (Freifield et al., (1937) in which 2,4-DNT was probably the major isomer present resulted in patients with red blood cells with Heinz bodies. This fact and other symptoms suggested the major physiological response to 2,4-DNT was methemoglobinemia. In studies of workers exposed to dinitrotoluene during World War II (McGee et al., 1942, 1947) indicated that patients suffered from headaches, weakness and lassitude, loss of appetite, nausea, vomiting, vertigo, pain or paresthesia in extremities, upper

abdominal discomfort, jaundice and liver tenderness.

Mammalian Toxicology of 2,4-Dinitrotoluene

Studies (Lee et al., 1975) on the absorption and distribution of C-14 labeled 2,4-DNT in rats showed the compound was readily absorbed after oral administration. The absorption was complete in 24 hours. The liver and kidney obtained significant amounts of radioactivity. Small amounts of radioactivity were also found in the other tissues including the brain, lungs, skeletal muscle and spleen. The tissue to plasma radioactivity ratio indicated that the nitrotoluenes and/or metabolites were readily taken into most tissues. Radioactivity was retained in some tissues especially the liver, kidneys and brain. Most of the absorbed radioactivity from the oral administration was excreted in the urine. The aromatic ring of 2,4-DNT remained in tact as confirmed by thin layer chromatography. Only a negligible amount of radioactivity was recovered in expired air.

Ellis et al.(1979) reported large variation in doses of 2,4-DNT required for a given toxic effect in mice, rats and dogs as shown in Table 3. In addition to effects on body weight and life span, 2,4-DNT was associated with methemoglobinemia (blood), ataxia and paralysis (central nervous system), degenerative lesions and hepatocellular carcinoma (liver), cystic changes and tumors (kidney), atrophy and aspermatogensis in the male, non functional follicles in the female, subcutaneous fibromas, mammary gland tumors and abnormal pigmentation.

Table 3.

SUMMARY OF TOXIC EFFECTS OF 2,4-DNT

IN MAMMALS

(Ellis et al., 1979)

Species	Dose (mg/Kg/day)	Toxicity
Dogs	0.2	no effect
	1.5	toxic to some
	10.0	toxic to all lethal to some
Rats	0.57-0.71	no effect
	3.9-5.1	toxic to some
	34.0-45.0	halved life span toxic to all
Mice	13.5	slightly toxic
	95.0	toxic to all
	900.0	halved life span

Aquatic Toxicology of 2,4-Dinitrotoluene

Sanford Research Institute (SRI) International (Menlo Park, CA) began to study the toxicity of 2,4-DNT under Army Medical Department Research and Development contract in 1977. The study of 2,4-DNT as a separate environmental toxin developed from the overall research effort on the toxicity of TNT wastewaters. Most of this literature review will be based on monthly progress reports on U.S. Army Medical Department contract research done by David W.H. Liu, SRI senior aquatic toxicologist and Howard C. Bailey, SRI fisheries biologist.

Monthly progress reports will be referenced by number, month and year.

Sanford Research Institute began to study the toxicity of TNT wastewater by developing a synthetic blend for bio-assays. This approach was used to avoid the costly prospect of examining the toxicological characteristics of the many components of TNT wastewater. The synthetic blend contained 2,4-DNT as the major component of TNT wastewater. Table 4 gives the complete mixture. Nonphotolyzed static acute tox-icity bioassays on the 17 compound synthetic blend resulted in the acute toxicity data shown in Table 5. The effect of pH on acute toxicity was examined with the 17 compound conden-sate blend and the bluegill sunfish as bioassay organism. The results are presented in Table 6. The results clearly show that toxicity of the 17 compound blend increased with decreasing pH. The same study examined the effect of water temperature on the toxicity of the 17 compound blend on blue-gill sunfish. The results are presented in Table 7. The data clearly indicates an increase in toxicity with increasing temperature. A second 22 compound condensate blend was develop-ed for further testing and is described in Table 8. Again, the major component of the 22 compound condensate blend is 2,4-DNT. Acute toxicity studies with nonphotolyzed synthetic condensate wastewater were conducted on early life stages of the fathead minnow. The results are presented in Table 9. As can be observed from the data, toxicity was greater in young fry and least in fertilized eggs. Exposure of waste-water, 2,4,6-TNT and 2,4-DNT, to uv light reduced their

Table 4.

COMPOSITION OF SYNTHETIC CONDENSATE WASTEWATER

17 COMPOUND BLEND

(SRI #26, OCT. 1977)

	COMPOUND	NOMINAL CONCENTRATION mg/l
1.	2,4-dinitrotoluene	50.72
2.	2,3-dinitrotoluene	1.52
3.	3,5-dinitrotoluene	1.51
4.	3,4-dinitrotoluene	1.51
5.	2,6-dinitrotoluene	22.01
6.	2,5-dinitrotoluene	1.22
7.	4-amino-3,5-dinitrotoluene	0.602
8.	4-amino-2,6-dinitrotoluene	1.83
9.	2-amino-4,6-dinitrotoluene	0.050
10.	5-amino-2,4-dinitrotoluene	2.45
11.	3,5-dinitrobenzene	13.58
12.	4,6-dinitroxylene	1.50
13.	2,4,6-trinitrotoluene	1.50
14.	0-nitrotoluene	0.075
15.	p-nitrotoluene	0.284
16.	3-methyl-2-nitrotoluene	0.030
17.	toluene	0.592

Table 5.

ACUTE TOXICITY DATA USING THE

17 COMPOUND CONDENSATE BLEND

(SRI #26, OCT. 1977)

SPECIES/ORGANISM	TEST	CONCENTRATION (mg/l)
Fathead minnow	96 hr LC_{50}	22.0
Bluegill sunfish	96 hr LC_{50}	7.2
Rainbow trout	96 hr LC_{50}	7.2
Channel catfish	96 hr LC_{50}	17.5
Daphnia magna	48 hr LC_{50}	22.7
Hyalella azteca	48 hr LC_{50}	22.7
Tanytarsus dissimilis	48 hr LC_{50}	47.0
Lumbriailus variegatus	48 hr LC_{50}	24.5
Navicula pelliculosa	4 day EC_{50}	9.2
	14 day EC_{50}	3.6
Selenastrum capricornium	4 day EC_{50}	3.2
	14 day EC_{50}	5.9
Microcystis aeruginosa	4 day EC_{50}	38.2
	14 day EC_{50}	3.0
Anabena flos-aquae	14 day EC_{50}	10.6

Table 6.

EFFECT OF pH ON ACUTE TOXICITY OF THE

17 COMPOUND CONDENSATE BLEND

IN THE BLUEGILL SUNFISH

(SRI #26, OCT., 1977)

pH	96 hr LC_{50} (mg/l)
6	5.3
7	6.1
8	7.1

Table 7.

EFFECT OF TEMPERATURE ON ACUTE TOXICITY OF THE

17 COMPOUND CONDENSATE BLEND

IN THE BLUEGILL SUNFISH

(SRI #26, OCT. 1977)

TEMPERATURE $^{\circ}$C	LC_{50}
15	7.0
20	6.6
25	3.9

Table 8.

COMPOSITION OF SYNTHETIC CONDENSATE WASTEWATER
22 COMPOUND BLEND
(SRI #26, OCT. 1977)

	COMPOUND	CONCENTRATION (%)
1.	2-nitrotoluene	0.09
2.	4-nitrotoluene	0.30
3.	1,3-dinitrobenzene	12.03
4.	2,6-dinitrotoluene	21.95
5.	2,5-dinitrotoluene	1.20
6.	2,4-dinitrotoluene	44.20
7.	2,3-dinitrotoluene	1.20
8.	3,5-dinitrotoluene	1.56
9.	3,4-dinitrotoluene	1.56
10.	2,4,6-trinitrotoluene	1.20
11.	1,3,5-trinitrotoluene	0.02
12.	2-amino-4,6-dinitrotoluene	0.06
13.	3-amino-2,4-dinitrotoluene	4.52
14.	3-amino-2,6-dinitrotoluene	3.61
15.	4-amino-2,6-dinitrotoluene	1.80
16.	4-amino-3,5-dinitrotoluene	0.60
17.	5-amino-2,4-dinitrotoluene	2.11
18.	2-amino-4-nitrotoluene	0.03
19.	toluene	0.60
20.	3-methyl-2-nitrophenol	0.03
21.	5-methyl-2-nitrophenol	0.09
22.	4,6-dinitro-m-xylene	1.20

Table 9.

TOXICITY OF 22 COMPOUND CONDENSATE BLEND TO

EARLY LIFE STAGES OF THE FATHEAD MINNOW

(SRI #26, OCT., 1977)

Life Stage	Toxicity	Concentration (mg/l)
Fertilized egg	96 hr LC_{50}	21.5
Fry 2 days	96 hr LC_{50}	4.1
7 days	96 hr LC_{50}	7.1
30 days	96 hr LC_{50}	8.1
60 days	96 hr LC_{50}	6.6
Adults	96 hr LC_{50}	11.3

toxicity. The pH of the wastewater during irradiation affected the color of the irradiated product but did not affect toxicity (SRI #29, Dec. 1977).

Bluegill sunfish were exposed to 1 mg/l of radiolabeled 2,4-DNT alone and as part of a 30 compound condensate under static bioassay conditions. Samples of the bluegill muscle and viscera were taken after 48 and 96 hours of exposure, and after three and ten days depuration following the 96-hour exposure. The results are presented in Table 10. Fish exposed under the conditions stated resulted in radioactivity in muscle and visceral samples of essentially zero after three days of depuration.

Table 10.

BIOCONCENTRATION OF 2,4-DINITROTOLUENE

IN THE BLUEGILL SUNFISH

(SRI #31, MAR., 1978)

Tissue	dpm/g Water	Bioconcentration Factor			
		48 hr Uptake	96 hr Uptake	3 day Depuration	10 day Depuration
Bluegill viscera	341	53	78	1	0
Bluegill muscle	341	4	4	0	0

Further progress on identification of components of 2,4,6-TNT wastewater resulted in the decision to prepare a thirty compound synthetic condensate blend. This blend is shown in Table 11. Again, 2,4-DNT was the major component of the blend (43.377%). Additions represent newly identified compounds in 2,4,6-TNT wastewater.

The decision was made (SRI #33, May, 1978) to start evaluation of 2,4-DNT separately by determining its acute toxicity to 12 species. The first static bioassays on the bluegill sunfish (SRI #34, June, 1978) resulted in the 24 hr LC_{50} in excess of 35 mg/l 2,4-DNT and the 96 hr LC_{50} of 15.7 mg/l with 95% confidence limits of 14.5 and 17.0 mg/l. The 48 hr LC_{50} for 2,4-DNT in four invertebrate species were determined. The results of this study are presented in Table 12. The investigators observed no toxic effects in _Hyalella azteca_ and _Lumbriculus variegatus_. The no effect concentration

Table 11.

FINAL RELATIVE CONCENTRATIONS FOR THE 30

COMPOUNDS CONDENSATE BLEND

(SRI #32, APR., 1978)

	Compound	Relative Concentration (percent)
1.	Toluene	0.59
2.	2-nitrotoluene	0.089
3.	4-nitrotoluene	0.295
4.	3-nitrotoluene	0.035
5.	4-nitrotoluene	0.027
6.	2-amino-4-nitrotoluene	0.091
7.	2-amino-6-nitrotoluene	0.030
8.	3-amino-4-nitrotoluene	0.080
9.	2-methyl-2-nitrophenol	0.035
10.	5-methyl-2-nitrophenol	0.094
11.	1,3-dinitrobenzene	11.803
12.	2,3-dinitrotoluene	1.18
13.	2,4-dinitrotoluene	43.377
14.	2,5-dinitrotoluene	1.18
15.	2,6-dinitrotoluene	21.541
16.	3,4-dinitrotoluene	1.475
17.	3,5-dinitrotoluene	1.534
18.	3,5-dinitroaniline	0.171
19.	1,5-dimethyl-2,4-dinitrotoluene	1.151
20.	2-amino-3,6-dinitrotoluene	0.089
21.	2-amino-4,6-dinitrotoluene	0.059

Table 11. (continued)

	Compound	Relative Concentration (percent)
22.	3-amino-2,4-dinitrotoluene	4.426
23.	3-amino-2,6-dinitrotoluene	3.541
24.	4-amino-2,6-dinitrotoluene	1.770
25.	4-amino-3,5-dinitrotoluene	0.59
26.	5-amino-2,4-dinitrotoluene	2.066
27.	2,4-dinitro-5-methylphenol	0.251
28.	1,3,5-trinitrobenzene	0.451
29.	2,3,6-trinitrotoluene	0.791
30.	2,4,6-trinitrotoluene	1.180

Table 12.

ACUTE TOXICITY OF 2,4-DNT TO

FRESHWATER INVERTEBRATES

(SRI #34, JUNE 1978)

Species	Pooled *48 hour LC_{50} and 95% confidence limits (mg/l)
Daphnia	38.3 (33.3-44.2)
Hyalella	>83.2
Tanytarsus	22.5 (19.6-25.7)
Lumbriculus	>83.2

*Quadruplicate test, five animals per replicate.

approached the aqueous solubility of the compound. The 96 hr LC_{50} of 2,4-DNT in bluegills was about 50% of that obtained in fathead minnows tested in 1975 (SRI #34, June, 1978).

Table 13 presents the (SRI #35, July, 1978) LC_{50} for 2,4-DNT in bluegills as affected by water temperature and hardness. Unlike the other compounds and compound mixtures associated with TNT wastewater, 2,4-DNT increased markedly in toxicity with increasing temperature. At 17°C, the 96 hr LC_{50} was 23.2 mg/l. At 21°C, the 96 hr LC_{50} decreased by about 50% to 12.8 mg/l. At 24°C, the 96 hr LC_{50} of 8.6 mg/l was about one-third of the LC_{50} obtained at 17°C. The water hardness did not affect the acute toxicity of 2,4-DNT significantly.

For channel catfish, the 24- and 96 hr LC_{50} for 2,4-DNT, based on static tests at 20°C, were 34.7 (33.0-36.4) and 28.8 (24.5-36.0) mg/l, respectively. The bluegill was the most sensitive fish species tested. The 96 hr LC_{50} in bluegills was 15.7 (14.5-17.0) mg/l, and the 96 hr LC_{50} in fathead minnows was 34.6 (33.0-36.3) mg/l (SRI #35, July, 1978).

Selenastrum capricornutum and Anabena flos-aquae were exposed to 2,4-DNT for 4- and 14-day EC_{50} determination (SRI #35, July, 1978). The 4- and 14-day EC_{50} with Selenastrum were 1.1 and 3.3 mg/l, respectively. The 14-day EC_{50} for Anabena was 10.2 mg/l. With Navicula pelliculosa (SRI #37, Sep., 1978) for the 14-day EC_{50} for 2,4-DNT was 8.s mg/l.

The LC_{50} for fish exposed to 2,4-DNT, determined under flow-through conditions with 95% confidence limits, are presented in Table 14 (SRI #38, Oct., 1978). As can be

Table 13.

EFFECT OF HARDNESS AND TEMPERATURE ON THE
ACUTE TOXICITY OF 2,4-DNT TO THE BLUEGILL SUNFISH
(SRI #35, JULY 1978)

Water Quality Parameter	Measured	LC_{50} and 95% Confidence Limits (mg/1)	
		24 Hour	96 Hour
Hardness (ppm $CaCo_3$)	47	33.0(31.4-34.6)	12.8(11.4-14.0)
	108	32.5(25,35)	18.8(17.0-20.7)
	240	31.5(25,35)	16.4(14.7-17.9)
Temperature (^oC)	17	27.7(24,32)	23.2(20.9-25.8)
	21	33.0(31.4-34.6)	12.8(11.4-14.0)
	24	16.4(14.6-18.5)	8.6(7.7-9.6)

Table 14.

LC_{50} DATA FOR FATHEADS AND CHANNEL CATFISH
EXPOSED TO 2,4-DNT UNDER FLOW-THROUGH CONDITIONS
(SRI #38, OCT., 1978)

Species	Exposure Condition	LC_{50} mg/1
Fathead	24 hr	36.5 (35.2-37.7)
	48 hr	36.5 (34.0-37.7)
	96 hr	36.5 (35.0-38.1)
	14 day	26.1 (24.0-28.2)
Catfish	24 hr	35.8 (25.7-46.0)
	48 hr	35.1 (34.0-36.2)
	96 hr	29.9 (27.3-33.0)
	14 day	15.6 (14.2-17.9)

observed, the toxicity of 2,4-DNT to fathead minnows and channel catfish was approximately the same under flow-through and static conditions.

Continued evaluation of the toxicity of 2,4-DNT to Lumbriculus variegatus (SRI #39, Nov., 1978) resulted in the LC_{50} at 24-, 96-, and 14-day of 99.5 (77.6-127.5), 50.7 (44.1-58.1) and 31.1 (27.9-34.2) mg/l, respectively. Lumbriculus variegatus was the least sensitive of the species tested under flow-through conditions.

The LC_{50} obtained for 2,4-DNT with rainbow trout as the bioassay organism are presented in Table 15 (SRI #41, Jan., 1979).

Table 15.
ACUTE TOXICITY OF 2,4-DNT TO RAINBOW TROUT
(SRI #41, JAN., 1979)

Exposure Time	LC_{50} mg/l
24 hr	>19.4
48 hr	>19.4
96 hr	14.0 (13.4-14.8)*
14 day	6.8 (6.6-7.1)*

*95% confidence interval

Sanford Research Institute began chronic toxicity studies on the fathead minnow in June, 1979. They found that (SRI #49, Sep., 1979) 2,4-DNT had no significant effect

on egg survival. The 2,4-DNT significantly reduced the number
of viable fry emerging from eggs exposed to 7.0 mg/l 2,4-DNT
in one test series. The 2,4-DNT had a significant effect on
the 30-day fry length at the 3 highest concentrations (1.75-
7.00 mg/l) in one series and had an effect on fry length at
the 2 highest concentrations (3.50-7.00 mg/l) in another test
series. There were significant effects on survival of fat-
head minnow fry exposed to 7.0 mg/l 2,4-DNT for 90 days (SRI
#54, Feb., 1980). A similar effect on survival was noted in
fry exposed to 7.0 mg/l for 150 days.

Subacute response data on 2,4-DNT is very limited.
Hartley et al., (1976) conducted a study to determine the
toxicity of M-1 propellant dust and dust components (2,4-DNT,
dibutylphthalate and diphenylamine) to freshwater organisms.
Acute toxicity (LC_{50}) data were similar to previously dis-
cussed studies. However, histopathological findings in the
fathead minnow were noted. At necropsy of fishes showing
sublethal responses, the musculature at sites of swelling was
hemorrhagic, particularly in the immediate vicinity of the
vertebral column. The hemorrhage was, in some cases, not
grossly visible depending on the proximity to the skin. The
skin and musculature were very translucent, and superficial
hemorrhage was visible in the swimming animal. Hemorrhage
was also present dorsal to the kidneys and, most prominently,
surrounding the dorsal aorta. Microscopically, the hemorrhage
first appeared in the vicinity of the dorsal aorta and dorsal
to the kidneys. Hemorrhage separated the pigmented layer

from the body wall dorsal to the kidneys. Hemorrhage was not present within the kidneys. Hemorrhage, radiating from the vicinity of the aorta, separated dorsal muscle bundles and fibers. Some sections showed red blood cells in the spinal canal, which may have entered through connective tissue bridges which join bony portions of the wall. In some cases, degeneration of the spinal cord was associated with the hemorrhage.

CHAPTER III

METHODOLOGY

METHODOLOGY

Phases of the Study

Phase I was to establish a flow-through exposure system that meets the requirements of the study and the United States Environmental Protection Agency standards for dynamic bioassay studies. A gas chromatographic method to monitor for 2,4-DNT in the flow-through bioassay system was developed. Design criteria to remove the waste 2,4-DNT from the exposure system via activated carbon sorption were determined.

Phase II was to establish baseline histological and physiological growth data on the bluegill sunfish. Organ systems evaluated were selected on the basis of previous studies of tissues with high sensitivity to low concentrations of environmental toxicants.

Phase III was to expose fish to sublethal concentrations of 2,4-DNT for 56 day (8 week) periods. Sublethal concentrations of 2,4-DNT were approximately 0.5, 5.0, 10.0, 15.0, 30.0, 40.0, and 60.0 percent of the LC_{50} for bluegills at 21°C (12.8 mg/l). Histological and physiological growth data were compared to controls and baseline data established in Phase II. Total body and organ specific distribution and elimination were determined via C^{14} labeled (ring C) 2,4-DNT.

Phase I

Flow-through System Design: The system consisted of 5 units as shown in Figure 1. Specifications for the flow-through system included the following:
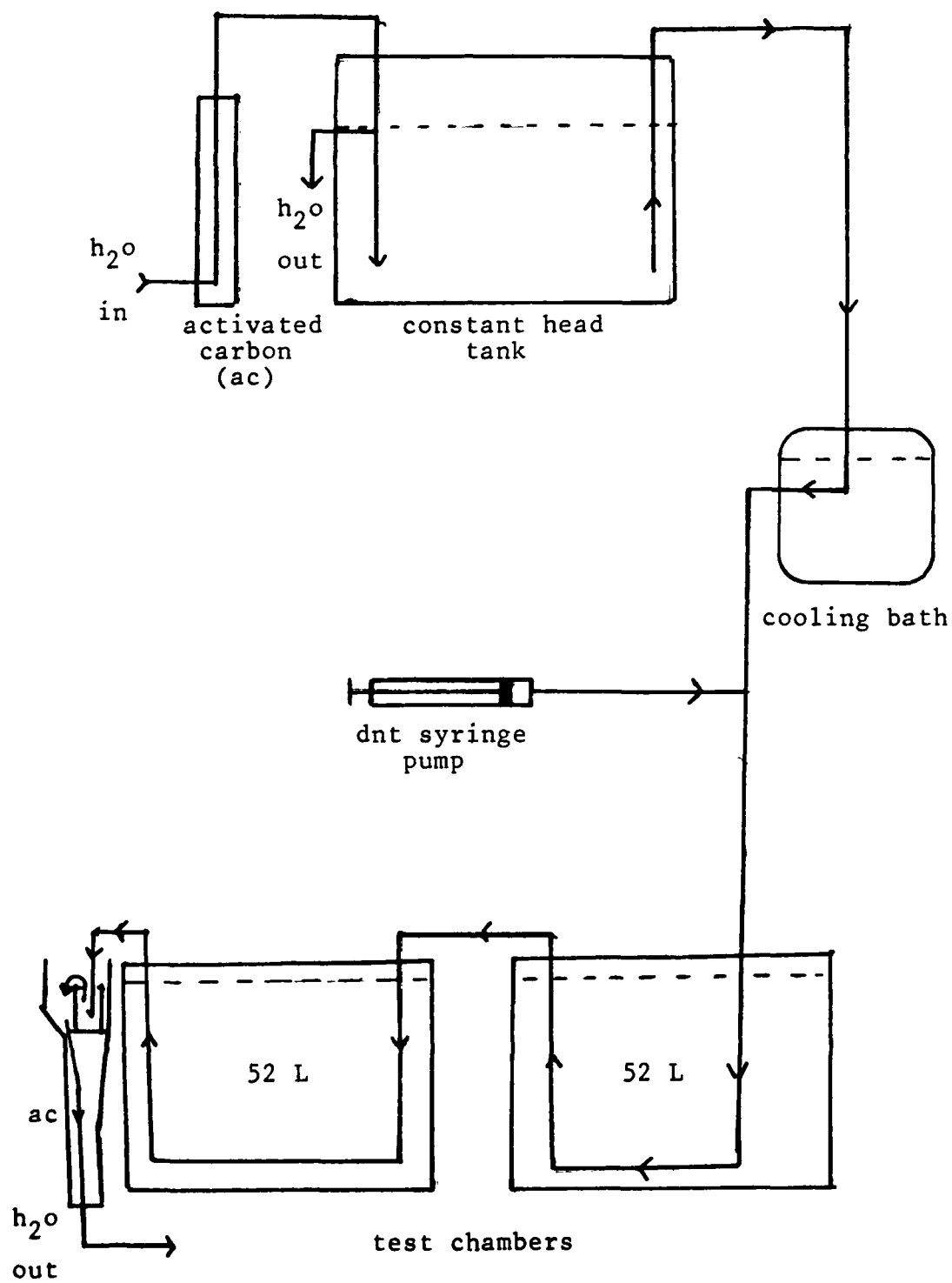
Figure 1. Unit Flow-Through System

1. There were six exposure units with a capacity for a series of 5 concentrations of toxicant and one control unit.

2. Temperature control was provided by the cooling bath unit. The temperature test range was 21°C ± 1°C.

3. Flow rate to each unit was approximately 300 ml/min and provided at least 4 to 5 complete turn-overs in the system per day.

4. Loading of the system with bioassay fish did not exceed 0.5 gm fish tissue per liter holding capacity of the system.

5. Harvard syringe injection pumps were used. The 2,4-DNT was injected in acetone carrier. The acetone was evaluated for toxicity. One syringe pump per unit was provided along with a back-up syringe injection system.

6. Activated carbon systems were provided to remove chlorine from incoming bioassay water as required.

7. Activated carbon was used to remove the 2,4-DNT from wastewater from the system at all times. Appendix A-1 outlines the design criteria developed and used in the system.

8. Fifty liter chambers were used for fish used in tissue studies and 50 liter chambers were for fish in growth studies.

<u>2,4-DNT Measurement</u>: Gas chromatography was used to analyze for the low concentrations of 2,4-DNT. The integrator was calibrated with a standard solution of 1.0 mg/l, 2,4-DNT. The solvent was hexane. The gas chromatograph with electron capture detector was used. The gas chromatograph was operated isothermally and the electron capture detector was maintained at $355^{o}C$. A 1.8 m glass column (2.0 vv i.d.) packed with 4 percent OV-101 on chromosorb GCQ (100/120 mesh) was used. The column temperature was $110^{o}C$ and the injection port temperature was $230^{o}C$. Five microliter samples in hexane were injected into the GC. The rational and extraction procedure for monitoring 2,4-DNT were completed in a separate study presented in Appendix A-7.

<u>Phase II and Phase III</u>

<u>Histological Preparations</u>: Tissue for histological examination was fixed with Bouin's fluid (75 ml picric acid; 25 ml 40% formalin; and 5 ml glacial acetic acid) or 10 percent formalin (1 part 40% formalin; 9 parts water), cleared in at least three changes of ethyl alcohol (70 percent, volume to volume), processed by an automatic tissue processor, paraffin embedded, frozen, sectioned (5 micron thick) and stained with Harris Hematoxylin and Eosin (HHE) or Periodic Acid Schiff (Hansen <u>et al</u>., 1974). All tissue was prepared for light microscopy. Serial sections at various levels were prepared from all major organs with special attention to gill, intestine, kidney, gonad, lateral line, liver, and muscle. All significant observations were documented with 3x5 inch black and white photographs.

Histological Baseline Data and Pathology: The following histological observations were made on control and exposed bluegill sunfish.

The Skin: Based on the subacute responses outlined in Appendix A-2, a complete description of fish integument was accomplished. This included the lateral line system and oral pharyngeal mucosa. Histological evaluation of the lateral line system included neuromast, pits and canals with cephalic branches.

The Digestive System: Based on subacute response studies outlined in Appendix A-3, histological descriptions of the fish gut included pharynx, esophagus, gastric mucosa, types of muscle in the intestine region and hind-gut. Pancreas and liver including zonal patterns were evaluated. In particular, fatty deposits and focal areas of necrosis in hepatic tissue were evaluated. Vacuolation of hepatic tissue was also evaluated.

The Respiratory System: Based on subacute response data in Appendix A-4, histological evaluation included the gill arches, branchial arches, lamellae, and capillary structure. Since toxicants in aquatic systems are likely to be absorbed osmotically, with most taken through the semipermeable gill and oral tissue, the gill was one of the most obvious tissues to look for subacute effects. Particular attention was devoted to looking for variation in mucus production, hypertrophy of gill filaments, hyperplasia of gill epithelium and coalescing of lamellae.

The Renal System: Based on subacute response data in
Appendix A-5, histological evaluation included the nephron
tubules, collecting duct system, glomerulus, convoluted
tubule, distal convoluted tubule, and mesonephric duct.
Particular attention was devoted to evaluation of necrosis
of renal tubular cells, reduction of renal lymphoid tissue,
dilation of renal tubules and vaculolation of renal cells.

The Nervous System: Histological evaluation included
the brain and spinal cord. The evaluation of lateral line
system has been previously discussed.

The Reproductive System: Based on the subacute re-
sponse data in Appendix A-6, a complete histological evalua-
tion of all reproductive tissue was accomplished. Particular
attention was directed to testicular or ovarian injury.

Carrier Evaluation: All 2,4-DNT was prepared in
acetone carrier. The maximum concentration of acetone did
not exceed 16.0 mg/l in the system. A separate single acetone
exposure series was conducted to ensure that no subacute ef-
fects resulted from this concentration of acetone. The
rational for this carrier concentration is given in Appen-
dix A-8. The single sublethal exposure series was tested for
subacute effects using 10.0 and 16.0 mg/l acetone and a
control. The unit exposure, sacrifice schedule and data
correlations were the same as those described for the 2,4-DNT
exposures discussed below.

Fish Holding and Acclimatization: Bluegill sunfish
2.0 to 5.0 cm long were obtained from ponds controlled by
the Louisiana Fish and Wildlife Department. Upon receipt of

fish, they were held in 55 gallon all glass aquaria provided with under-sand filters and high capacity charcoal/fiber filters. The first day the fish were not fed. They were treated with 0.01 mg/l methylene blue to reduce the possibility of fungus infection due to crowded laboratory conditions. Fish were fed an amount of high protein food representing approximately 5 percent of their body weight three times daily. Excess food was removed after feeding. Fish were held for at least two weeks before use in the flow-through system bioassays.

Sublethal Exposure Series: Each series was an eight week exposure consisting of the sublethal concentrations of 2,4-DNT to be tested and a control (i.e., 6 units of the flow-through exposure system in use). The sublethal concentrations used were 0.05 mg/l, 0.50 mg/l, 1.0 mg/l, 2.0 mg/l, 4.0 mg/l, 5.0 mg/l and 8.0 mg/l 2,4-DNT.

Sample Unit Exposure: There were a total of 75 blue-gill sunfish in each unit. The fish lengths were 2 to 5 centimeters. Twenty-five fish were used in the growth studies and 50 fish in histology studies. Bioassay conditions were $21^{\circ}C \pm 1^{\circ}C$, pH 7.4, and dissolved oxygen concentration of approximately 8.0 mg/l. Daily measurements included temperature, pH and dissolved oxygen concentration. The 2,4-DNT concentration was measured weekly.

Sacrifice Schedule and Growth Measurement: Five fish were sacrificed weekly for histopathology and fixed as previously detailed. Fish used in the growth portion of the study were selected so that initial weights were approximately

0.5 grams. The weights of the fish in the growth chambers were determined weekly. The fish were calmed prior to weighing by using the anesthetic 2-phenoxyethanol (PE), $C_6H_5OCH_2$ CH_2OH, (Idler et al., 1961; Bell, 1964; Sedev et al., 1963 and McBride et al., 1963). To weigh the fish quickly and minimize handling stress, the flow-through system was stopped and PE was added to the growth chambers at the dose of 1.5 ml per gallon (Bell, 1964). After the fish were sufficiently calm (no response to gentle touch), they were quickly weighed on a tare weight balance and returned to the growth chambers. The normal flow to the growth chambers was then restored.

Data Correlations: All significant histopathological observations were documented with photographs. Testing of zero order, first order, second order and LOG-LOG growth models was accomplished using linear regression. The constants developed were tested for significance using Tukey's Paired Comparison Procedure (Box et al., 1978). The growth models were selected on the basis of best fit (R-squared value). A significance level of $\alpha=0.05$ or $\alpha=0.01$ was used to determine the validity of the procedure as a toxicity test and to determine which concentrations of 2,4-DNT produce an effect on bluegill growth.

2,4-DNT Uptake/Depuration in Bluegills: Four 52 liter chambers with 3.0 mg/1 2,4-DNT (carrier and the C^{14} ring labeled compound) were prepared. Activity was approximately 240 nanocuries per liter. A recirculating pump and fiber filter was available to remove large particulate matter if

required. The chambers were sealed with foil to reduce volitalization of the compound. Tanks were blacked about to reduce photolytic action on the DNT.

Two hundred fish were added to the four tanks. They were exposed to 3.0 mg/l 2,4-DNT for 14 days and there were seven days depuration. Each day the following samples were taken:

1. One milliliter of water for liquid scintillation counting (LSC) was taken from each tank.

2. A total of six fish were taken from the four tanks. Two of the fish were used for whole body DNT analysis. The remaining four fish were prepared to provide samples of liver-pancreas, stomach-ascending descending intestine, striated muscle, brain, gill tissue and renal tissue with associated connective tissue.

Each week a 500 ml sample of water from each tank was taken to determine 2,4-DNT concentration by gas chromatography. The safety precautions included use of an activated carbon trap on the gas chromatograph.

There were two complete water changes during the study to minimize loss of 2,4-DNT via bacterial breakdown or photolysis. The wastewater was contained in polyethylene trash cans with recirculating pumps. The water was continuously recirculated through activated carbon until all 2,4-DNT

including the C^{14} labeled DNT was adsorbed. The final waste was approximately one pound of activated carbon.

Disposal of and handling of labeled compound was handled according to established procedures at Tulane University Medical Center.

After the two week exposure to 3.0 mg/l 2,4-DNT, the remaining fish were transferred to tanks without 2,4-DNT for the one week depuration. The sacrifice schedule was continued as previously described.

The tissue was solubilized with Protosol (New England Nuclear) and Aquasol (New England Nuclear) was the LSC solution. One milliliter water samples were counted directly using 15 ml of Aquasol. Quenching versus efficiency and quenching versus weight of tissue standard curves were determined according to standard LSC methods. Modeling of up-take and loss curves from whole fish and specific tissues were attempted. All samples were counted on a Beckman LS-150 with Cesium^{137} internal standard.

CHAPTER IV

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Purity of 2,4-DNT Bulk Supply

The 2,4-DNT used for the bioassays was purchased in bulk from ICN K&K Laboratory, Plainview, New York. A gas chromatographic method was developed to separate and identify all six isomers of DNT. Analyses were performed using a Hewlett Packard 5830A gas chromatograph equipped with an electron capture detector (ECD) and 1885A terminal integrator. A six foot glass column was packed with two percent OV101 on chromosorb GCQ (100-120 mesh) prepared by Hewlett Packard Co. Optimum operating conditions for separating the six isomers of DNT were as follows:

Column Temperature - $110^{\circ}C$

Injection Port Temperature - $225^{\circ}C$

ECD Temperature - $300^{\circ}C$

Carrier Gas (5% methane, 95% argon) 20 cc/min

Typical retention times for an optimal mixture of isomers and minimum detection for the ECD are summarized in Table 16. The chromatogram for Table 16 is shown in Figure 2.

Although there was peak overlap in some cases, the resolution was sufficient for accurate analyses. However, the proportion by weight of 2,3-DNT must not exceed 25 percent of the 2,4-DNT to obtain adequate resolution of these two isomers. This restriction would not be a problem in dealing with actual environmental samples because very little 2,3-DNT would be present compared to 2,4-DNT. The isomers present in the bulk supply were 2.4% 2,6-DNT and 97.6% 2,4-DNT by weight.

Table 16.

RETENTION TIMES AND MINIMUM DETECTION TO

ELECTRON CAPTURE DETECTION OF SIX DNT ISOMERS*

DNT Isomer	Retention Time Minutes	Minimum Detection to ECD Micrograms
2,6-DNT	16.55	62.5×10^{-6}
2,5-DNT	20.73	62.5×10^{-6}
2,3-DNT	23.93	62.5×10^{-6}
2,4-DNT	25.43	25.0×10^{-5}
3,5-DNT	27.43	25.0×10^{-5}
3,4-DNT	33.83	12.5×10^{-5}

*solvent hexane
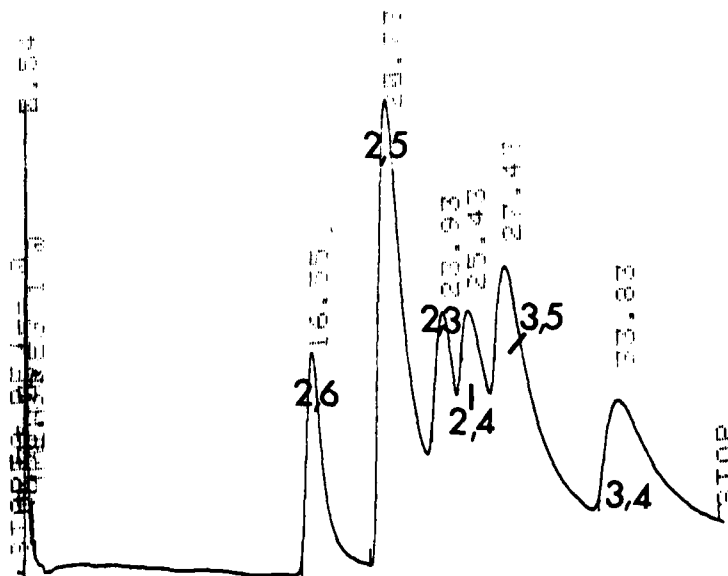
Figure 2. Typical Chromatogram for a Mix of Six DNT Isomers.

Acetone Carrier Exposure Series and 2,4-DNT Exposure Series A and B

The purposes of the Acetone Carrier Exposure Series were to determine if the concentrations of acetone in the system would influence the growth of bluegill sunfish or induce tissue lesions. The concentrations selected were 10.0 mg/l, 16.0 mg/l acetone and a control.

The purposes of Series A 2,4-DNT exposures were to determine the range of concentrations of 2,4-DNT that would result in subacute growth response effects and subacute tissue lesions in the bluegill sunfish. The concentrations of 2,4-DNT selected for Series A exposures were 0.5 mg/l, 2.0 mg/l, 5.0 mg/l and 8.0 mg/l 2,4-DNT plus a control.

The purposes of Series B exposures were to establish the upper and lower limits of the concentration of 2,4-DNT that produce an effect on fish growth and to evaluate the use of growth models in the study of subacute response. The development of subacute tissue lesions was also evaluated in Series B. The concentrations of 2,4-DNT selected based on the results of Series A exposures were 0.05 mg/l, 0.50 mg/l, 1.00 mg/l, 2.00 mg/l, 4.00 mg/l 2,4-DNT and a control.

2,4-DNT Concentrations for Test Series

The results of the weekly monitoring of 2,4-DNT concentrations in exposure Series A and Series B are detailed in Appendix C-1 and C-2, respectively. The mean concentrations of 2,4-DNT ± standard deviations (S.D.) in Series A were 0.504 ± 0.057 mg/l, 2.093 ± 0.164 mg/l, 5.106 ± 0.165 mg/l and 8.053 ± 0.164 mg/l. The mean 2,4-DNT concentrations

for Series B were 0.052 ± 0.007 mg/l, 0.492 ± 0.028 mg/l, 1.070 ± 0.077 mg/l, 2.06 ± 0.161 mg/l and 4.04 ± 0.257 mg/l. For a flow-through system the measured 2,4-DNT concentrations were close to those desired. Therefore, throughout the narrative, the attempted 2,4-DNT concentrations previously discussed will be used on all figures and tables.

Dissolved Oxygen Concentrations, pH and Temperature

The results of the daily monitoring for dissolved oxygen in exposure Series A and Series B are presented in Appendices C-3 and C-4, respectively. The mean dissolved oxygen concentrations ±S.D. for Series A and Series B were 8.01 ± 0.309 mg/l and 7.82 ± 0.335 mg/l, respectively. The results of daily monitoring for pH (-LOG concentration of hydrogen ion) for Series A and Series B are detailed in Appendices C-5 and C-6, respectively. The mean pH's ±S.D.'s for Series A and Series B were 7.58 ± 0.175 and 7.56 ± 0.181, respectively. The results of daily monitoring for temperature in exposure Series A and Series B are found in Appendices C-7 and C-8, respectively. The means ±S.D. for exposure Series A and Series B were 21.06 ± 0.509 °C and 21.12 ± 0.493 °C, respectively.

Fish Growth Models for Subacute Response to 2,4-DNT

A typical growth data set for Series A is the control data shown in Table 17. The remaining data sets for the series are detailed in Appendices C-9 through C-12. A typical growth data set for Series B is the control data shown in Table 18. The remaining data sets for the series are detailed

Table 17.

GROWTH DATA FOR SERIES A – WEIGHT IN GRAMS

CONTROL

Fish No.	Days											
	0	5	10	15	20	25	30	35	40	45	50	55
1	0.51	0.57	0.55	0.61	0.64	0.73	0.71	0.78	0.81	0.87	0.98	1.21
2	0.53	0.59	0.61	0.64	0.60	0.72	0.76	0.77	0.89	0.91	0.99	1.18
3	0.50	0.56	0.60	0.62	0.68	0.71	0.75	0.80	0.85	0.99	0.98	1.11
4	0.52	0.58	0.63	0.67	0.70	0.67	0.76	0.84	0.80	0.92	1.10	1.20
5	0.51	0.54	0.61	0.65	0.64	0.65	0.79	0.73	0.79	0.86	1.07	1.09
6	0.50	0.59	0.59	0.69	0.65	0.73	0.72	0.71	0.80	0.96	1.04	1.07
Mean	0.512	0.572	0.598	0.647	0.652	0.702	0.748	0.772	0.823	0.918	1.027	1.140
S.D.*	0.012	0.019	0.027	0.030	0.035	0.034	0.029	0.047	0.039	0.050	0.051	0.061

*Standard deviation

Table 18.

GROWTH DATA FOR SERIES B - WEIGHT IN GRAMS

CONTROL

Fish	Days								
No.	0	7	14	21	28	35	42	49	56
1	0.50	0.53	0.57	*	*	*	*	*	*
2	0.53	0.45	0.55	0.73	0.80	*	*	*	*
3	0.49	0.54	0.50	0.66	0.72	0.83	0.91	0.98	1.10
4	0.50	0.57	0.65	0.65	0.71	0.74	0.81	0.97	1.29
5	0.52	0.55	0.56	0.67	0.71	0.84	0.81	0.94	1.19
6	0.54	0.54	0.58	0.62	0.65	0.87	0.79	0.99	1.17
7	0.51	0.57	0.66	0.58	0.78	0.83	0.85	0.93	1.10
8	0.54	0.50	0.70	0.65	0.77	0.79	0.86	0.95	1.19
9	0.49	0.54	0.51	0.67	0.69	0.77	0.87	0.98	1.12
10	0.57	0.57	0.61	0.63	0.73	0.84	0.85	1.09	1.10
11	0.50	0.53	0.61	0.66	0.81	0.76	0.90	0.99	0.99
12	0.50	0.51	0.62	0.76	0.78	0.71	0.93	0.92	1.19
13	0.47	0.56	0.63	0.65	0.73	0.75	0.85	0.99	1.16
14	0.55	0.51	0.62	0.64	0.61	0.74	0.85	0.96	1.12
15	0.51	0.59	0.62	0.71	0.71	0.80	0.83	1.10	1.26
16	0.45	0.56	0.61	0.71	0.71	0.69	0.89	1.02	1.31
17	0.49	0.56	0.70	0.69	0.68	0.82	0.86	0.86	1.21
18	0.50	0.46	0.61	0.59	0.81	0.87	0.89	0.96	1.23
19	0.50	0.58	0.59	0.62	0.72	0.85	0.87	0.92	1.24
20	0.45	0.49	0.57	0.69	0.73	0.80	0.88	0.98	1.19
21	0.47	0.56	0.60	0.75	0.77	0.76	0.86	0.84	1.23
22	0.50	0.55	0.59	0.67	0.76	0.80	0.86	1.01	1.19
23	0.54	0.63	0.60	0.60	0.73	0.83	0.89	0.98	1.25
24	0.48	0.55	0.55	0.60	0.69	0.70	0.89	0.97	1.19
25	0.49	0.55	0.59	0.55	0.57	0.79	0.87	0.99	0.95
Mean	0.504	0.542	0.600	0.656	0.724	0.790	0.864	0.970	1.173
S.D.**	0.030	0.040	0.048	0.053	0.059	0.053	0.033	0.058	0.087

*Fish death
**Standard deviation

in Appendices C-13 through C-17. Based on a general evalua-
tion of the data and the literature, four models were selected.
They were zero order, first order, second order and LOG-LOG
models. The growth model most commonly used for fast growing
(juvenile) fish is the exponential (first order) model.
Ricker (1958, 1971) defined the model as:

$$\frac{W_T}{W_O} = e^{gT}$$

where:

W_T = weight of the fish at time, T

W_O = weight of the fish at T = 0

g = constant

He also proposes the use of <u>relative</u> <u>growth</u> <u>rate</u> (h) which was
defined as:

$$h = \frac{\text{weight change for a specific time}}{\text{initial weight at the beginning of time period}}$$

The next growth model to be considered was the LOG-LOG
model. This model is derived from the observation that the
weight (w) of a fish usually varies as some power of length
(Ricker, 1958, 1971), (Nielson and Schoch, 1980), and is
expressed as:

$$w = al^b$$

where:

l = length of fish

a = initial weight at length=o or y-intercept

b = constant

Second order reactions are common in chemical and
biological reactions. The equation proposed by Metcaff and

Eddy (1972) was evaluated and is expressed as:

$$\frac{1}{C_T} = \frac{1}{C_o} = K_2 T$$

where:

C = concentration of reactant

C_o = concentration of reactant at time, $T = 0$

C_T = concentration at time, T

K_2 = constant

T = time

Finally, the zero order (linear) model was considered and is commonly expressed as:

$$W_T = mT + W_o$$

where:

W_T = weight at time, T

W_o = initial weight ($T = 0$)

m = constant

T = time

These four growth models are summarized in Table 19 and converted to a uniform nomenclature for the purposes of further comparison of subacute toxicity data. A visual presentation (Figure 3) of weight versus time, cumulative h (relative growth rate) versus time, and h versus time was developed. The figure illustrates a hypothetical case of a population of juvenile fish that gain the same weight in a specificed time period but conform to each model in Table 19.

Table 19.

GROWTH MODELS WITH UNIFORM NOMENCLATURE

FOR SUBACUTE TOXICITY

EQUATION (NUMBER) ORDER	EQUATION	LOG TRANSFORMED	CONSTANT GROWTH RATE	GRAPHICAL TECHNIQUE
(4) ZERO	$W_T = mT + W_o$	N/A	$m = \dfrac{W}{T}$	W vs T
(1) FIRST	$W_T = W_o e^{a_1 T}$	$\mathrm{LOG}_e\, W_T = \mathrm{LOG}_e\, W_o + a_1 T$	$a_1 = \dfrac{\mathrm{LOG}_e\, W_2 - \mathrm{LOG}_e\, W_1}{T_2 - T_1}$	$\mathrm{LOG}_e\, W$ vs T
(3) SECOND*	$\dfrac{1}{W_T} = \dfrac{1}{W_o} + a_2 T$	N/A	$a_2 = \left[(T_2 - T_1)(W_2 - W_1) \right]^{-1}$	$\dfrac{1}{W}$ vs T
(2) LOG-LOG	$W_T + W_o T^n$	$\mathrm{LOG}\, W_T = \mathrm{LOG}\, W_o + n\, \mathrm{LOG}\, T$	$n = \dfrac{\mathrm{LOG}\, W_2 - \mathrm{LOG}\, W_1}{\mathrm{LOG}\, T_2 - \mathrm{LOG}\, T_1}$	$\mathrm{LOG}\, W$ vs $\mathrm{LOG}\, T$

TERMS:

W_T = weight at time, T   T = Time (T)

W_o = weight at time, T=0 T_2 = Time T=2

W_2 = weight at time, T=2 T_1 = Time T=1

W_1 = weight at time, T=1   m = zero order constant (i.e., gm day^{-1})

a$_1$ = 1st order constant (i.e., day^{-1})

a$_2$ = 2nd order constant (i.e., gm-day)

n = LOG-LOG model constant

*$T \leq -\dfrac{1}{W_o a_1}$, if T exceeds $-\dfrac{1}{W_o a_1}$ the model becomes invalid for fish growth studies.

Figure 3.   Different Measures of Growth Based on Four
            Growth Models.
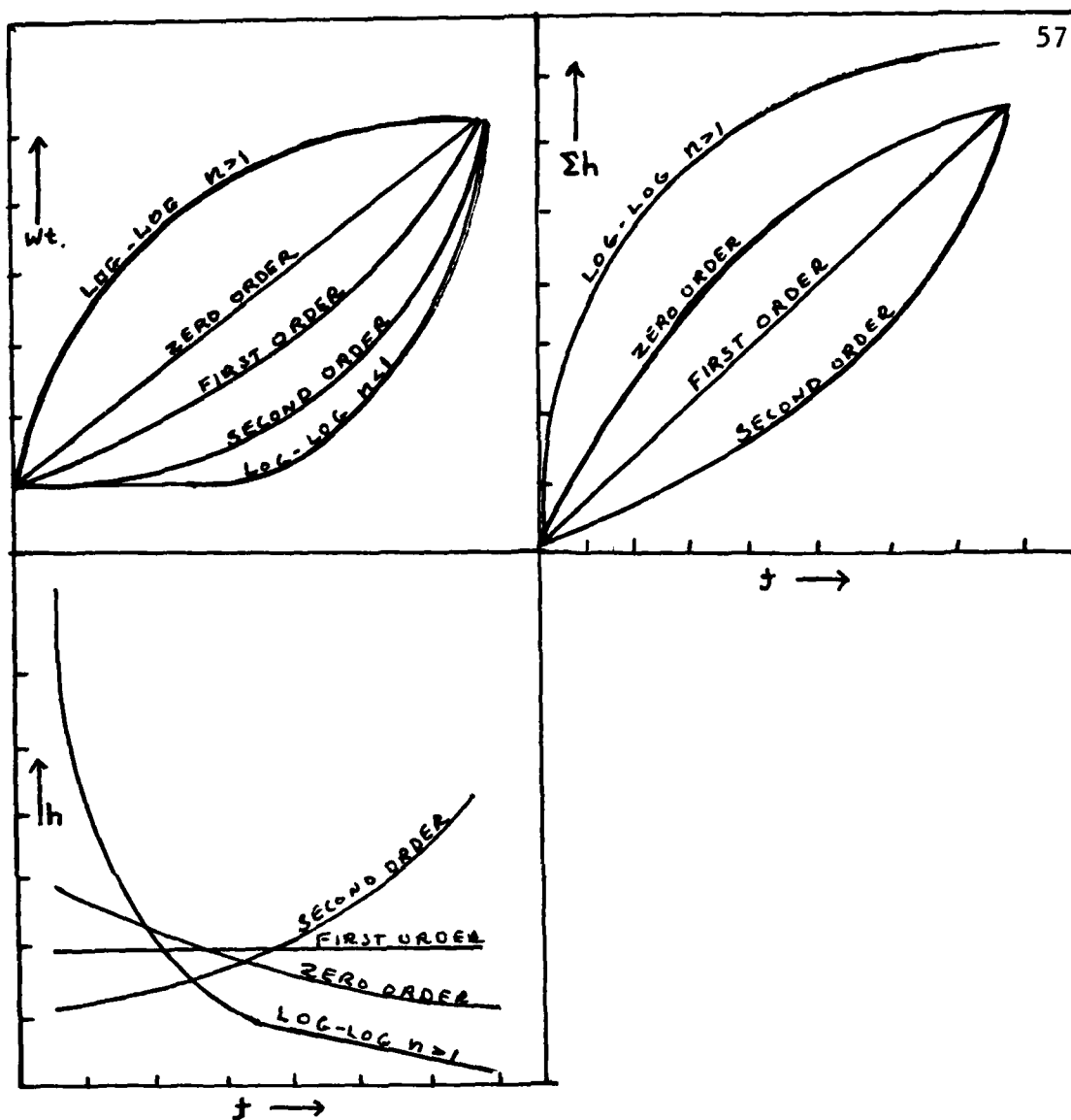
Wt = weight in grams

h = relative growth rate

Σh = growth rate(cummulative relative)

t = time in days

Acetone Carrier Series

The growth data for the acetone carrier exposure series for the control, 10.0 mg/l and 16.0 mg/l acetone exposure groups are found in Appendices B-1, B-2 and B-3, respectively. Growth constants (a_1) based on the first order model are detailed in Appendix B-4. As demonstrated by the high R-squared values (94.7-95.6 percent) for the exposure groups, the first order model is adequate to determine the effect of the acetone carrier on bluegill growth.

Based on Tukey's paired comparison procedure, the growth constants for all possible pairs (Appendix B-5) of exposure groups are not significantly different at the $\alpha=0.01$ (error rate) level. It is concluded that the maximum levels of acetone that may be present in the flow through system will not influence the outcome of the studies to determine the effect of 2,4-DNT on bluegill growth.

Results of the histological studies of the fish exposed to acetone (control, 10.0 mg/l and 16.0 mg/l) are detailed in Appendix B-6. Mild hyperplasia of gill lamellae and evidence of high hepatic glycogen were found in both controls (20%) and acetone exposure groups (20-40 percent). These histological effects were observed from day 49-56 of the exposure series. In conclusion, the effect of using acetone as a carrier for the 2,4-DNT toxicity study has been determined to be negligible. These two responses are not considered pathological responses and will be discussed in detail in the histopathology section.

Subacute Effect of 2,4-DNT on Fish Growth Series A Exposures

Series A exposures were accomplished with a small sample size of six fish per 2,4-DNT concentration (control, 0.50 mg/l, 2.00 mg/l, 5.00 mg/l and 8.00 mg/l) to determine the range for subacute response to be used in Series B exposures. The cumulative relative growth rate (Σh) versus time for the series is graphically presented in Figure 4. The relative growth rate (h) and Σh data for the series are detailed in Appendices C18 and C-19, respectively. An analysis of Figure 4 indicates that several of the four previously developed growth models might apply. Regression analysis of the growth data (Appendices C-9 through C-12, Table 17) resulted in series of equations based on the four models along with R-squared values shown in Table 20. The constants for each case/concentration of 2,4-DNT along with the standard deviations (S.D.) are presented in Table 21.

These data indicate that there was no significant growth of fish exposed to 5.00 mg/l and 8.00 mg/l 2,4-DNT for the eight week period. Visual inspection of Figure 4, low constants and R-squared values in Tables 20 and 21 confirm this conclusion. Lack of significant growth of fish under adverse environmental conditions has been observed by Pessah and Powles (1974) with pumpkinseed sunfish at 5°-10°C water temperatures. Horning and Pearson (1973) demonstrated weight loss in small mouth bass at temperatures in excess of 35°C water temperature. Hence, lack of growth or weight loss is a common occurrence in freshwater fish under adverse conditions.
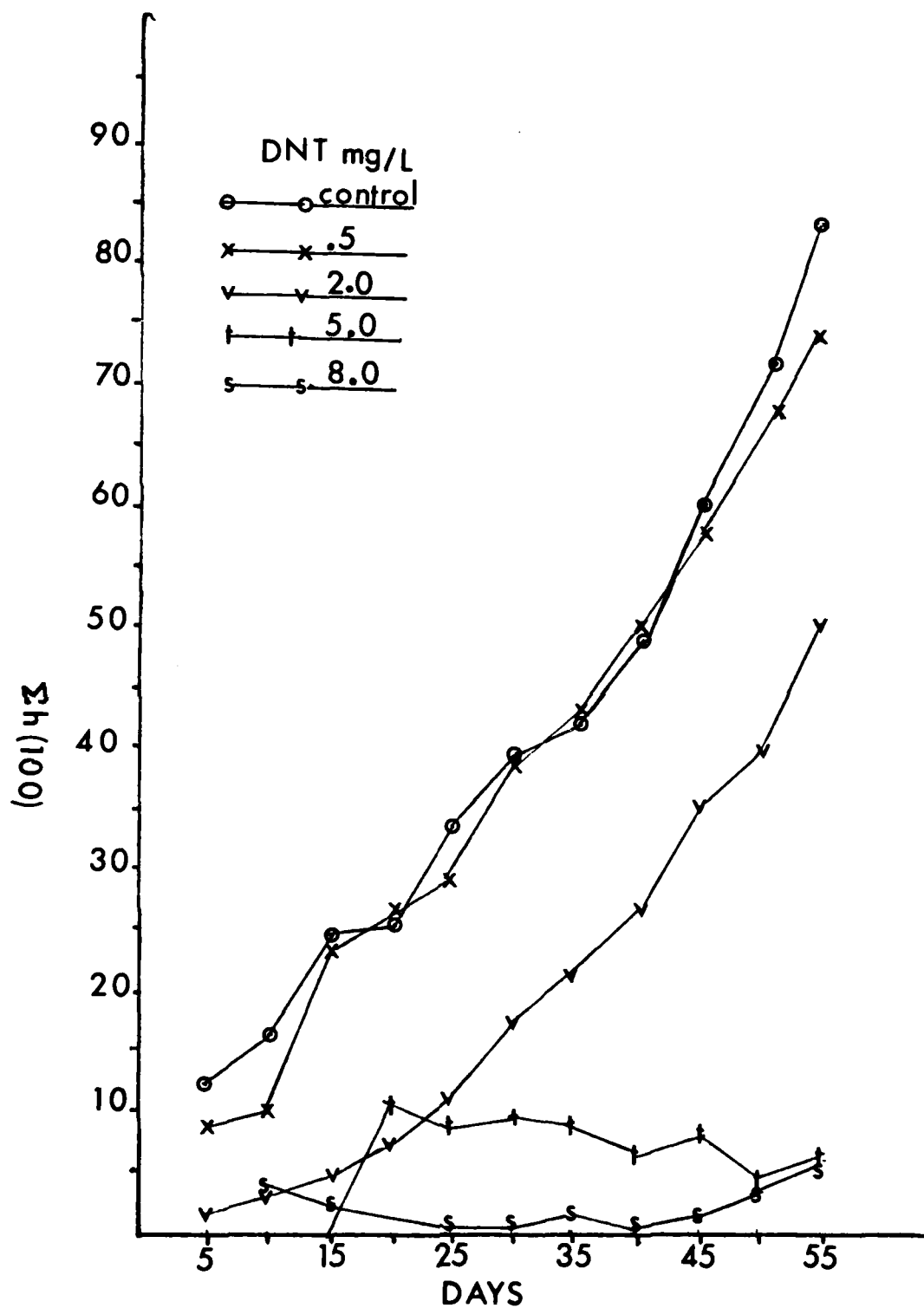
Figure 4. Mean Cumulative Relative . owth Rate for Eight
Week Exposure to 2,4-DNT Series A.

Table 20.

SERIES A MODEL EQUATIONS AND R-SQUARED VALUES

2,4-DNT mg/l	ORDER			
	Zero	First	Second	LOG-LOG
Control	W=0.479+0.0102T	lnW=-0.666+0.0132T	$\frac{1}{W}$=1.88-0.0178T	LOG W = -0.487+0.266 LOG T
R^2	89.6%	93.9%	95.0%	75.7%
0.5	W=0.481+0.0088T	lnW=-0.685+0.0123T	$\frac{1}{W}$=1.93-0.0176T	LOG W = -0.493+0.256 LOG T
R^2	92.0%	93.7%	93.0%	80.2%
2.0	W=0.465+0.0053T	lnW=-0.740+0.0085T	$\frac{1}{W}$=2.06-0.0139T	LOG W = -0.456+0.174 LOG T
R^2	88.4%	91.3%	93.2%	71.3%
5.0	W=0.491+0.0011T	lnW=-0.717+0.0022T	$\frac{1}{W}$=2.06+0.0045T	LOG W = -0.373+0.0643 LOG T
R^2	20.9%	20.7%	20.2%	26.1%
8.0	W=0.496+0.0003T	lnW=-0.701+0.0007T	$\frac{1}{W}$=2.02+0.0013T	LOG W = -0.316+0.0142 LOG T
R^2	9.0%	8.6%	8.2%	4.9%

Table 21.

SPECIFIC CONSTANTS AND STANDARD DEVIATIONS (X100) AT 21°C FOR

SERIES A AND SERIES B EXPOSURES TO 2,4-DNT

Series and Conc. 2,4-DNT (mg/l)	gm day^{-1} Zero Order Constant	ln gm day^{-1} First Order Constant	(gm day)$^{-1}$ Second Order Constant	LOG gm LOG day^{-1} LOG-LOG Constant
SERIES A				
Control	1.02±0.04	1.32±0.04	-1.78±0.05	26.58±1.86
R^2	89.6%	93.9%	95.0%	75.7%
0.5	0.89±0.03	1.23±0.04	-1.76±0.06	25.60±1.73
R^2	92.0%	93.7%	93.0%	80.2%
2.0	0.53±0.03	0.85±0.03	-1.39±0.05	17.45±1.58
R^2	88.4%	91.3%	93.2%	71.3%
5.0	0.11±0.03	0.22±0.06	-0.45±0.12	6.43±1.60
R^2	20.9%	20.7%	20.2%	26.1%
8.0	0.04±0.02	0.07±0.03	-0.13±0.06	1.42±0.86
R^2	9.0%	8.6%	8.2%	4.9%

Table 21. (continued)

Series and (mg/l) Conc. 2,4-DNT	gm day^{-1} Zero Order Constant	ln gm day^{-1} First Order Constant	(gm day)$^{-1}$ Second Order Constant	LOG gm LOG day^{-1} LOG-LOG Constant
SERIES B				
Control	1.10±0.03	1.44±0.03	-1.99±0.04	33.79±1.22
R^2	88.7%	92.3%	91.8%	80.3%
0.05	0.99±0.02	1.33±0.02	-1.86±0.03	30.67±1.06
R^2	91.4%	94.5%	94.1%	81.5%
0.50	0.88±0.02	1.25±0.03	-1.84±0.04	28.98±1.21
R^2	88.8%	90.9%	90.7%	75.8%
1.00	0.81±0.02	1.19±0.03	-1.81±0.04	28.96±1.21
R^2	87.9%	90.2%	90.6%	75.9%
2.00	0.54±0.02	0.07±0.02	-1.41±0.03	21.72±0.93
R^2	88.0%	89.8%	90.3%	76.7%
4.00	0.14±0.01	0.27±0.01	-0.53±0.02	5.95±0.30
R^2	87.9%	88.1%	88.2%	72.7%

However, this does not account for the high variability of
the growth data in the 5.0 mg/l and 8.0 mg/l exposures and
the resultant poor fit of growth models. Different tolerances
for 2,4-DNT among the bluegill may account for the high vari-
ability of the growth data (Brett, 1971).

Further analysis of the data by computing Tukey's (Box
et al., 1978) paired comparison procedure for the constants
for each of the four growth models were conducted using alpha
(total error rate) values of 0.01 and 0.05. Statistically
different pairs of constants were identified. Results of
these analyses for all concentrations of 2,4-DNT (plus control)
for the zero order model (Appendix C-20), first order model
(Table 22), second order model (Table 23) and LOG-LOG model
(Appendix C-21) are presented.

The first and second order models fit the data best
with R-squared values greater than 90 percent for the control,
0.05 mg/l 2,4-DNT and 2.00 mg/l 2,4-DNT exposures. The growth
constants declined as the concentration of 2,4-DNT increased
in both first and second order models. Considering the first
order model, Tukey's confidence intervals indicate that all
possible pairs of constants were statistically different at
alpha value of 0.01. Therefore, there were statistically
different growth constants among all the test groups both
when the exposed were compared to the control and when the
exposed groups were compared to each other.

In the case of the second order model, the conclusions
were the same as those based on the first order model with
one exception. The growth constants for the control -0.05 mg/l

Table 22.

TUKEY'S PAIRED COMPARISON PROCEDURE FOR CONSTANT (a_1) SERIES A EXPOSURES, FIRST ORDER MODEL

α = 0.05

Confidence interval = $a_1 \pm 0.0706$

	Control	0.50	2.00	5.00	8.00
		concentration (mg/l) 2,4-DNT			
Mean (a_1)	1.32	1.23	0.85	0.22	0.07
		S*(0.09)†	S(0.47)	S(1.10)	S(1.25)
			S(0.38)	S(1.01)	S(1.16)
				S(0.63)	S(0.78)
					S(0.15)

α = 0.01

Confidence interval = $a_1 \pm 0.0875$

	Control	0.50	2.00	5.00	8.00
		concentration (mg/l) 2,4-DNT			
Mean (a_1)	1.32	1.23	0.85	0.22	0.07
		S(0.09)	S(0.47)	S(1.10)	S(1.25)
			S(0.38)	S(1.01)	S(1.16)
				S(0.63)	S(0.78)
					S(0.15)

*Statistically significant difference between pairs
†Actual difference between pairs

Table 23.

TUKEY'S PAIRED COMPARISON PROCEDURE FOR CONSTANT (a_1)
SERIES A EXPOSURES, SECOND ORDER MODEL

$\alpha = 0.05$

Confidence interval = $a_2 \pm 0.1242$

	concentration (mg/l) 2,4-DNT				
	Control	0.50	2.00	4.00	8.00
Mean (a_2)	-1.78	-1.76	-1.39	-0.45	-0.13
		NS**(0.02)†	S*(0.39)	S(1.33)	S(1.65)
			S(0.37)	S(1.31)	S(1.63)
				S(0.94)	S(1.26)
					S(0.32)

$\alpha = 0.01$

Confidence interval = $a_2 \pm 0.1539$

	concentration (mg/l) 2,4-DNT				
	Control	0.50	2.00	4.00	8.00
Mean (a_2)	-1.78	-1.76	-1.39	-0.45	-0.13
		NS(0.02)	S(0.39)	S(1.33)	S(1.65)
			S(0.37)	S(1.31)	S(1.63)
				S(0.94)	S(1.26)
					S(0.32)

 *Statistically significant difference between pairs
**Not statistically significant difference between pairs
 †Actual difference between pairs

pair were not significantly different at the alpha equal 0.01 or 0.05 total error rate.

The zero order model and the LOG-LOG model did not fit the data as well as the first and second order models as evidenced by lower R-squared values. However, analysis of the constants by Tukey's paired comparison procedure resulted in conclusions similar to those indicated by the first and second order models.

On the basis of the conclusions developed by data modeling of Series A exposures, the concentration range of 2,4-DNT that produces a subacute effect on the growth characteristics of juvenile bluegill sunfish was less than 0.5 mg/l 2,4-DNT (lower limit) and less than 5.0 mg/l (upper limit). Therefore, 0.05 mg/l, 0.50 mg/l, 1.00 mg/l, 2.00 mg/l and 4.00 mg/l 2,4-DNT were selected for testing in the Series B exposures.

Series B Exposures

Series B exposures were accomplished with a sample size of 25 fish per 2,4-DNT concentration. The purposes of this series were to determine the lower and upper limits of the concentration of 2,4-DNT that produces an effect on fish growth and to evaluate the validity of the models in determining the subacute effects of DNT on growth in an eight week toxicity test. The cumulative relative growth rate (Σh) versus time for the series is graphically presented in Figure 5. The relative growth rate (h) and Σh data for the series are detailed in Appendices C-22 and C-23, respectively. A visual examination of Figure 5 indicates that the data should fit some of the four growth models. Regression analysis of the
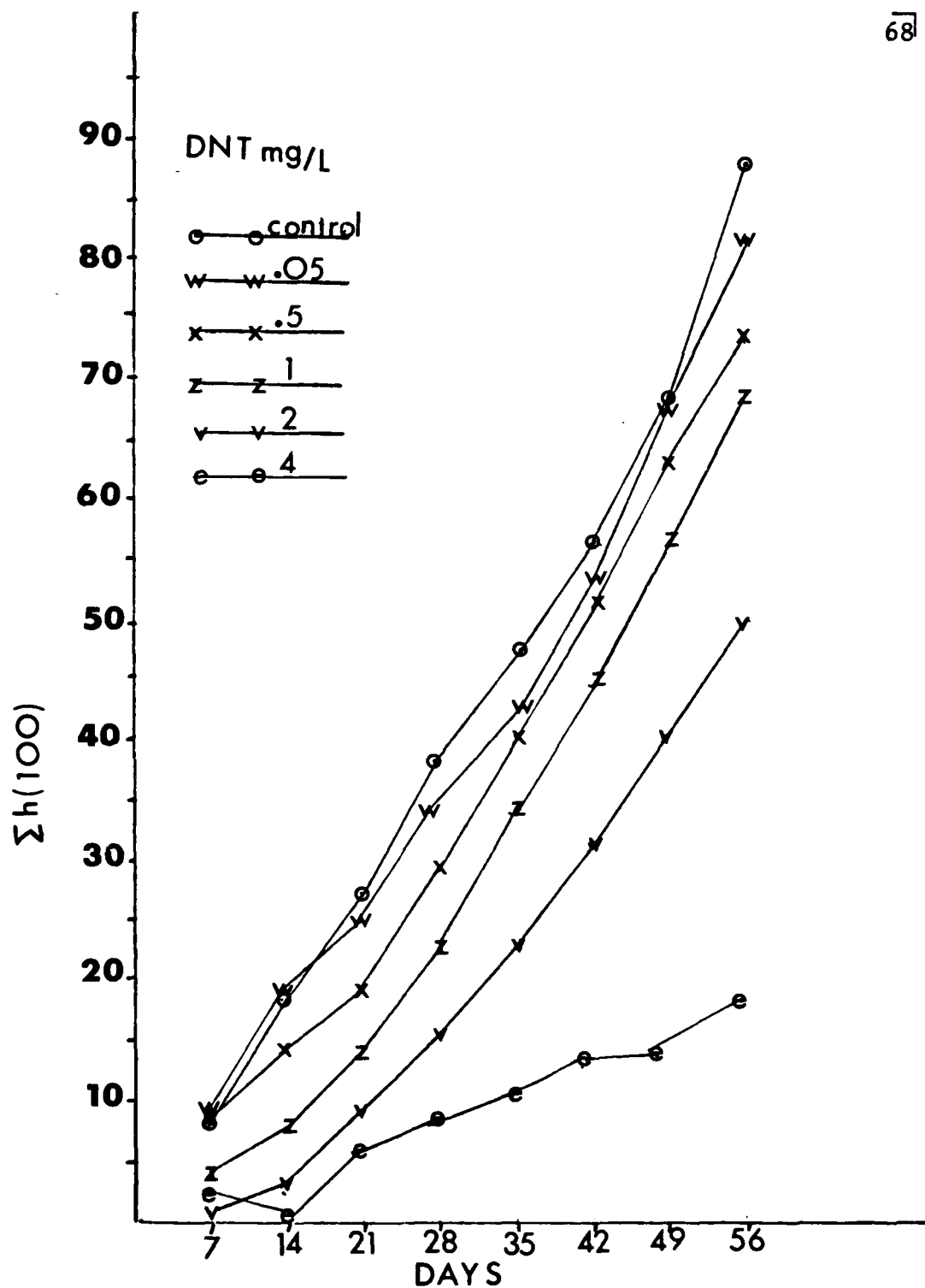
Figure 5. Mean Cumulative Relative Growth Rate for Eight Week Exposure to 2,4-DNT, Series B.

growth data (Appendices C-13 through C-17, Table 18) resulted in a series of equations based on the four models along with R-squared values shown in Table 24. The constants for each concentration of 2,4-DNT along with the standard deviation (S.D.) are given in Table 21.

Visual inspection of Figure 5 and high R-squared values indicate that the data fits the first and second order models. Growth specific constants generally decrease as the concentration of 2,4-DNT increases in the exposure series. Further analyses of the data by computing Tukey's paired comparison procedure for the constants for each of the growth models were conducted as previously described for Series A exposures. Results of these analyses for all concentrations of 2,4-DNT (plus control) for the zero order model (Appendix C-24), first order model (Table 25), second order model (Table 26) and LOG-LOG model (Appendix C-25) are presented.

The first and second order models fit the data best with R-squared values greater than 88.0% throughout the 2,4-DNT test concentration range. Considering the first order model, Tukey's confidence intervals indicate that all possible pairs of constants (which represent growth responses to 2,4-DNT concentrations) were statistically different at alpha equals 0.01. Considering the second order model, the results were the same with two exceptions. At alpha equal 0.05 there was no statistical difference between the constants for the 0.05-0.50 mg/1 DNT exposure groups. At alpha equal 0.01, there are no statistical differences between the constants for the 0.05-0.50 mg/1 2,4-DNT and the 0.5-1.00 mg/1 test groups.

Table 24.

SERIES B MODEL EQUATIONS AND R-SQUARED VALUES

2,4-DNT mg/l	ORDER			
	Zero	First	Second	LOG-LOG
Control	$W=0.452+0.0110T$	$lnW=-0.716+0.0144T$	$\frac{1}{W}=1.97-0.0198T$	$LOG\ W=-0.596+0.338\ LOG\ T$
R^2	77.6%	92.3%	91.8%	80.3%
0.05	$W=0.463+0.0099T$	$lnW=-0.704+0.0133T$	$\frac{1}{W}=1.96-0.0186T$	$LOG\ W=-0.562+0.307\ LOG\ T$
R^2	91.4%	94.5%	94.1%	81.5%
0.5	$W=0.451+0.0088T$	$lnW=-0.740+0.0125T$	$\frac{1}{W}=2.03-0.0184T$	$LOG\ W=-0.564+0.290\ LOG\ T$
R^2	88.8%	90.9%	90.7%	75.8%
1.0	$W=0.440+0.0081T$	$lnW=-0.766+0.0119T$	$\frac{1}{W}=2.09-0.0181T$	$LOG\ W=-0.585+0.290\ LOG\ T$
R^2	87.9%	90.2%	90.6%	75.9%
2.0	$W=0.461+0.0054T$	$lnW=-0.747+0.0087T$	$\frac{1}{W}=2.07-0.0141T$	$LOG\ W=-0.518+0.217\ LOG\ T$
R^2	88.0%	89.8%	90.3%	76.7%
4.0	$W=0.478+0.0014T$	$lnW=-0.737+0.0027T$	$\frac{1}{W}=2.09-0.0053T$	$LOG\ W=-0.367+0.0595\ LOG\ T$
R^2	87.9%	88.1%	88.2%	72.7%

Table 25.

RESULTS OF TUKEY'S PAIRED COMPARISON PROCEDURE FOR CONSTANT (a_1) – SERIES B EXPOSURES FIRST ORDER MODEL

$\alpha = 0.05$

Confidence interval = $a_1 \pm 0.0197$

	Concentration (mg/l) 2,4-DNT					
	Control	0.05	0.50	1.00	2.00	5.00
mean(a_1)	1.44	1.33	1.25	1.19	0.87	0.27
Significant pairs matrix		S*(0.11)†	S(0.19)	S(0.25)	S(0.57)	S(1.17)
			S(0.08)	S(0.14)	S(0.46)	S(1.06)
				S(0.06)	S(0.38)	S(0.98)
					S(0.32)	S(0.92)
						S(0.60)

$\alpha = 0.01$

Confidence interval = $a_1 \pm 0.0233$

	Concentration (mg/l) 2,4-DNT					
	Control	0.05	0.50	1.00	2.00	5.00
mean(a_1)	1.44	1.33	1.25	1.19	0.87	0.27
Significant pairs matrix		S(0.11)	S(0.19)	S(0.25)	S(0.57)	S(1.17)
			S(0.08)	S(0.14)	S(0.46)	S(1.06)
				S(0.06)	S(0.38)	S(0.98)
					S(0.32)	S(0.92)
						S(0.60)

*Statistically significant difference between pairs
†Actual difference between pairs

Table 26.

RESULTS OF TUKEY'S PAIRED COMPARISON PROCEDURE FOR
CONSTANT (a_2) – SERIES B EXPOSURES
SECOND ORDER MODEL

$\alpha = 0.05$

Confidence interval = $a_2 \pm 0.0275$

	Concentration (mg/l) 2,4-DNT					
	Control	0.05	0.50	1.00	2.00	4.00
mean(a_2)	-1.99	-1.86	-1.84	-1.81	-1.41	-0.53
Significant		S*(0.13)†	S(0.15)	S(0.18)	S(0.58)	S(1.46)
cant						
pairs			NS**(0.02)	S(0.05)	S(0.45)	S(1.33)
matrix						
				S(0.03)	S(0.43)	S(1.31)
					S(0.40)	S(1.28)
						S(0.88)

$\alpha = 0.01$

Confidence interval = $a_2 \pm 0.0325$

	Concentration (mg/l) 2,4-DNT					
	Control	0.05	0.50	1.00	2.00	4.00
mean(a_2)	-1.99	-1.86	-1.84	-1.81	-1.41	-0.53
Significant		S(0.13)	S(0.15)	S(0.18)	S(0.58)	S(1.46)
cant						
pairs			NS(0.02)	S(0.05)	S(0.45)	S(1.33)
matrix						
				NS(0.03)	S(0.43)	S(1.31)
					S(0.40)	S(1.28)
						S(0.88)

 *Statistically significant difference between pairs
**Not statistically significant difference between pairs
 †Actual difference between pairs

The R-squared values for the zero order model range
from 87.9-91.4 percent. Based on Tukey's confidence intervals
with alpha equal 0.01 identical conclusions as those reached
with the first order model apply. Conclusions are not drawn
from the LOG-LOG model due to poor fit (R-squared 72.7-81.5%).

Discussion of Growth Results

There are several problems of model fit in toxicity
studies and interpretation of the data regarding conclusions
about the toxicity of 2,4-DNT. When fish growth is used as a
measure of toxicity, several environmental factors must be
controlled in the laboratory. They are those factors that
influence the genetic potential for bluegills reaching a
characteristic size under the most favorable conditions. The
presumption is that under less favorable conditions fish will
be smaller than the maximum physiologically attainable for
the species (Bond, 1979). Also, it is commonly agreed that
fish growth occurs in stanzas that are determined by maturity,
seasons, food availability, crowding, temperature and many
other physiological factors (Ricker, 1971; Gerking, 1966; and
Price, 1977). The potential variability due to these factors
were minimized in this study by maintaining constant tempera-
ture ($21^{\circ}C$), space requirements (0.5 gm tissue/1) and using
Age 0 juvenile bluegills of approximately 0.5 grams each at
the onset of each exposure series. Two week temperature
acclimatization in the flow-through exposure unit also reduced
variability (Pessah and Powles, 1974).

Significant growth in bluegills occurs over a broad
range of temperatures in the natural environment from a few

degrees below the upper lethal limits (35.6 to 37.3°C) to
temperatures of approximately 20°C (Beitinger and Magnuson,
1979). Anderson (1958) reported that there is little or no
growth in bluegills below 10-13°C. Growth was more rapid at
20.5°C than at 25.5°C when two groups of bluegills were
placed on the same daily rations (Ricker, 1949).

Laboratory studies on the bluegill where temperature
was controlled along with feeding were conducted by Beitinger
and Magnuson (1979) and Lemke (1977) in which they used the
exponential (first order) model to study bluegill growth and
thermoselection. The first order model has been previously
defined. As shown in Table 27, the first order specific
growth constants obtained in this study provide information
concerning bluegills grown in the laboratory at 21°C. The
growth constants developed in this research are in the range
shown in Beitinger and Magnuson's (1979) and Lemke's (1977)
work. It is proposed that the variability is less due to
careful selection of age group and size at the onset of the
exposures (Carlander, 1977).

The first and second order growth constants indicate
reduced growth rates as the concentration of 2,4-DNT increases
(Table 21). This expected response in sunfish exposed to
inhibitory temperatures or toxic conditions has been observed
by Pessah and Powles (1974). They observed that pumpkinseed
bluegills had reduced growth rates during 16 week exposures
to 5-10°C water. Lemke and Mount (1973) using 13 mg/l alkyl
benzene sulfonate, Van Valin et al. (1968) using mirex,
Gilderhus (1966) using sodium arsenate and Anderson et al.

Table 27.

SPECIFIC FIRST ORDER GROWTH CONSTANTS* (CONTROL DATA)
FOR BLUEGILLS FED AD LIBITUM UNDER CONSTANT
TEMPERATURE CONDITIONS. (Values are means ±S.D.
for this study and means and ranges for the cited
sources; sample sizes are in parentheses.)

Temperature °C	56 days growth (This study)	10 days growth (Beitinger and Magnuson, 1979)	30 days growth (Lemke, 1977)
21	1.44±0.03(25)		
	1.32±0.04(6)		
24			1.71(12)
			0.64-3.19
25		2.36±0.30(5)	
28		2.30±0.50(5)	1/92(11)
			1.02-2.56
32			1.81(12)
34		1.50±0.32	1.89(12)
			1.26-2.74

*100X change in ln (wet weight) day^{-1}

(1966) using heptachlor observed reduced growth rates in blue-
gills.

The first order growth constants were significant at
all concentrations of 2,4-DNT tested when exposed groups were
compared to the controls and to each other. Based on these
results, (Table 25) the threshold 2,4-DNT concentration for
subacute growth response in bluegills was approximately 0.05
mg/l 2,4-DNT. Considering the actual difference in constants

between the control and 0.05 mg/l exposure group, the actual threshold may be a little less than 0.05 mg/l. The upper limit of 2,4-DNT concentration that produces a subacute growth response without high mortalities was between 4.0 mg/l and 5.0 mg/l 2,4-DNT. This conclusion was based on the fact that very little and no bluegill growth occurs at 4.0 mg/l and 5.0 mg/l 2,4-DNT, respectively. Also, mortalities at 8.0 mg/l 2,4-DNT are very high due to closeness to the LD50.

The same conclusions concerning threshold and upper limit 2,4-DNT concentrations can be made on the basis of the second order model. However, using the second order growth constants to evaluate the data lends to different conclusions concerning comparison of exposed groups with each other (Table 26). The growth constants (α = 0.01) for the 0.05-0.50 mg/l 2,4-DNT and 0.5-1.00 mg/l 2,4-DNT pair are not significantly different. This statistical evaluation raised the question if the graded growth responses from the 0.05-1.0 mg/l 2,4-DNT exposure groups are biologically significant. This reservation did not apply if the first order model growth constants were used.

The data fits both the first and second order models equally well from a statistical view. Considering the limitations of the second order model previously discussed and the predominance of first order models in fisheries data, conclusions regarding the graded growth response of bluegills to subacute concentrations of 2,4-DNT are accepted by the author. However, the potential advantages of the second order model are that it would allow for better fit of growth response

data when the toxicant produced a more pronounced growth lag phase (Figure 3). The zero order and LOG-LOG models are clearly inferior in this study. A zero order model could be useful when using more mature and slower growing fish. The LOG-LOG model could be useful for a population of very rapid growing fish (n = >1) or very slow growing fish (n=<1) when they are exposed to subacutely toxic conditions (Figure 3).

This portion of the research clearly demonstrated the threshold concentration for 2,4-DNT (0.05 mg/1) with a graded decreased growth response up to 5.0 mg/1 2,4-DNT. Using a first and second order model, the laboratory procedure used could be considered as a bioassay or quantative toxicity test under the stated laboratory conditions.

Histopathology of Subacute Response to 2,4-DNT

Liver

The normal histology of the bluegill liver is shown in Figure 6. The liver has a poorly defined lobular structure. It is composed of layered, two cell thick laminae of hepatocytes (parenchymal sheets) separated by sinusoids. Blood flows from the hepatic portal vein and hepatic artery through the sinusoids to the central veins which empty into the hepatic vein. These basic structures are similar to the livers of channel catfish (Grizzle and Rogers, 1976), rainbow trout (Anderson and Michum, 1974) and menhaden (Cahn, 1975). Bile canaliculi are not visible with the light microscope. Grizzle and Rogers, (1976) showed using electron microscopy that they did not have their own walls but were extensions of intercellular spaces between hepatocytes.
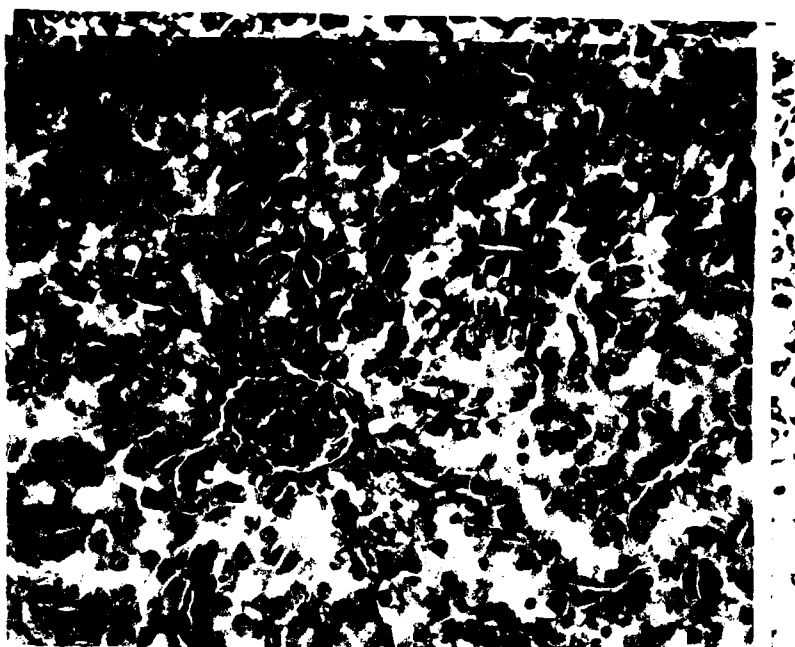
Figure 6.   Liver (400X) Bouin's, H&E. Normal control liver.
Laminae of hepatocytes (a) around a central
vein (b) with associated sinusoids (c) filled with
blood cells (d).
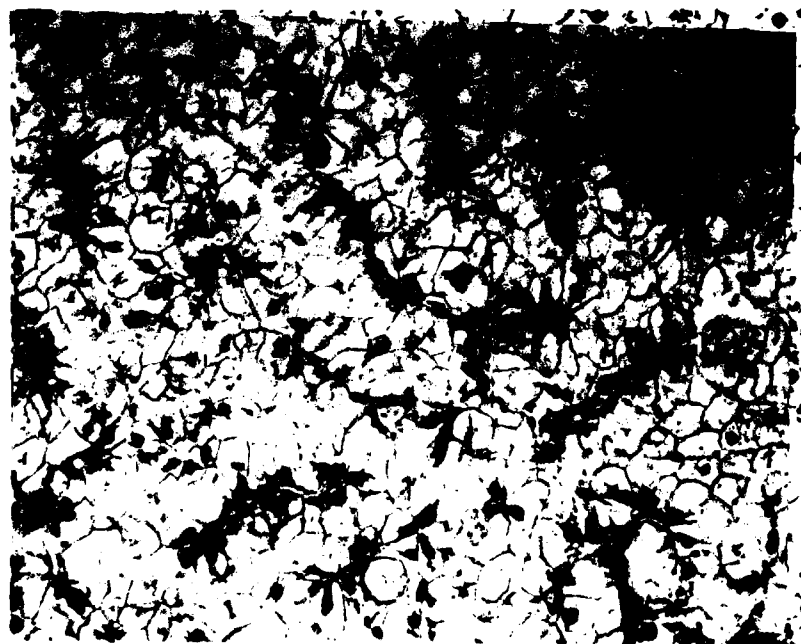
Figure 7.   Liver (400X) Bouin's, H&E. Exposed to 0.50 mg/1
2,4-DNT for 56 days. Considerable glycogen extrac-
tion of hepatocytes but otherwise similar to
control sections.

The amount of glycogen or lipid (fat) stored in hepatocytes determines to a great degree how liver sections appear after sectioning and H&E staining. The importance of liver glycogen and lipid metabolism was reviewed by Ralph (1978). Glucose as well as some galactose and fructose, which the liver converts to glucose is incorporated into the polysaccharide glycogen or transformed into fat by liver cells. Some fat synthesized by the liver may be stored there, but most of it is normally transported by the blood to adipose tissue cells. Dietary differences in the amount of stored glycogen were clearly illustrated by standard histological techniques in rat liver (Cardell et al., 1973). In fish, hepatocytes are often filled with glycogen, giving a very vacuolated, pale appearance in hematoxylin and eosen (H&E) sections (Ashley, 1975).

Figure 6 and Figure 7 show the range of normal hepatis responses in bluegill's exposed to low concentrations of 2,4-DNT and controls. They reflect the glycogen content and resultant extraction of the glycogen in the section preparation and staining procedure. Figure 6 is a control liver section with much basophilic cytoplasmic granulation. There are a few vacuoles (areas of extracted glycogen) and poorly resolved cell boundries. This liver is representative of the lower glycogen content hepatocytes and resultant low glycogen extraction in the tissue preparation process. Figure 7 represents a higher glycogen content in hepatocytes and higher glycogen extraction rate in tissue preparation. There are many vacuoles (areas of glycogen extraction) and well defined cell boundries.

Sinusoids have little blood and were not as dilated as those in Figure 6. Similar ranges of cytoplasmic granulation and vacuolation were noted by Cahn (1975) upon examination of livers of atlantic menhaden. In some livers there was much cytoplasma granulation, fewer vacuoles, basophilic cytoplasm and poorly defined cell boundries. In others there was little granulation, much vacuolation, acidophilic cytoplasm and well defined cell boundries. This study and Cahn's (1975) demonstrated uniform hepatic tissue appearance throughout individual control fish livers.

The livers examined for Series A and B control, 0.05 mg/l, 0.50 mg/l, 1.0 mg/l, 2.00 mg/l 2,4-DNT exposures demonstrate the lower glycogen levels indicated by the histological pattern shown in Figure 6. From days 49-56 of the exposures, 10-40 percent of livers examined demonstrate the higher glycogen level indicated by the histological picture shown in Figure 7. These results are summarized for Series A and B exposures in Tables 28 and 29, respectively. It is assumed that the vacuoles in Figure 7 are not filled with lipids since lipid vacuoles are usually smooth-edged (Couch, 1975). Hinton and Pool (1976) found that in sections stained with toluidine blue, cytoplasmic staining was more intense near the nucleus while areas at the periphery exhibited little or no staining. He found that this effect was due to glycogen loss due to processing and occasional lipid droplets. It is the opinion of this writer that the hepatic responses in this study as demonstrated on Figures 6 and 7 were not a pathological response to 2,4-DNT concentrations previously detailed. Although glycogen

Table 28.

ESTIMATED TIME OF ONSET OF PATHOLOGICAL EFFECTS IN SERIES A***

Type of Lesion/Effect	Control	0.50	mg/l 2,4-DNT 2.0	5.0	8.0
LIVER					
glycogen depletion	49*(10%)**				
lipid accumulation		56(30%)	49(20%)	56(10%)	49(20%)
necrotic foci					49)20%)
SPLEEN					
hemosiderin inclusions					
KIDNEY (TRUNK)					
atypical tubules				49(10%)	49(20%)
tubule necrosis				49(10%)	49(20%)
LATERAL LINE					
atypical neuromast cells				56(20%)	
necrotic epithelium				56(20%)	
GILL					
mild hyperplasia		45(10%)	35(10%)		
hypertrophy			42(20%)		
severe hyperplasia				49(10%)	56(20%)

*Day of sacrifice (estimated time of onset)
**Percent of fish with lesion out of the total examined for that week (i.e., 10% = 1 fish for Series A).
***Ten fish examined per week for each concentration of 2,4-DNT tested

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Table 29.

ESTIMATED TIME OF ONSET OF PATHOLOGICAL EFFECTS IN SERIES B***

Type of Lesion/Effect	Control	0.05	mg/l 2,4-DNT 0.50	1.00	2.00	4.00
LIVER						
glycogen extracted						
lipid accumulation	56*(20%)**	56(20%)	49(20%)	56(20%)	56(40%)	
necrotic foci						56(20%)
SPLEEN						
hemosiderin inclusions						45(40%)
KIDNEY (TRUNK)						
atypical tubules						
tubule necrosis						
LATERAL LINE						
atypical neuromast cells						
necrotic epithelium						
GILL						
mild hyperplasia	56(20%)	56(40%)	49(40%)	56(20%)		
hypertrophy			56(20%)		49(20%)	
severe hyperplasia						

*Day of sacrifice (estimated time of onset)
**Percent of fish with the lesion out of the total examined for that week (i.e., 20% = 1 fish for Series B).
***Five fish examined per week for each concentration of 2,4-DNT tested.

82
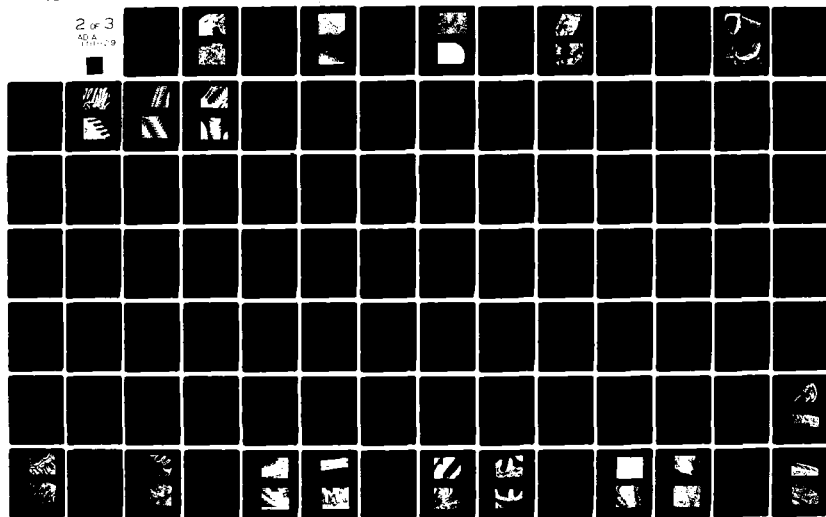
depletion due to diet (Cardell et al., 1973) and laboratory
holding under control or experimental exposure (Couch, 1975)
have been demonstrated, the broad range of liver response
demonstrated in this study (as evidenced by Figures 6 and 7)
contraindicates glycogen depletion as a uniform response to
laboratory diet or experimental exposure in this study. It
is the conclusion of the author that the liver variations
observed for fish exposed to 0.05 mg/l, 0.5 mg/l, 1.0 mg/l
and 2.0 mg/l generally fall within a normal range since the
morphological integrity of the tissue is not affected. How-
ever, it must be noted that vacuolated livers indicative of
glycogen extraction in tissue processing appear late in the
exposure period and in a slightly higher percentage of cases
among some exposed groups. This issue can only be resolved
when more information is available concerning what constitutes
a normal liver histology in the bluegill sunfish under a wide
range of environmental conditions.

Figure 8 and Figure 9 demonstrate large smooth-edged
vacuoles characteristic of lipid deposits in livers from
bluegills exposed to 4.0 mg/l, 5.0 mg/l and 8.0 mg/l 2,4-DNT.
The onset of fatty change (Table 28 and Table 29) was from
49 to 56 days exposure with 10 to 20 percent of the fish
evaluated demonstrating lipid deposits. Although no frozen
sections using oil red O to positively confirm the lipid
content of the vacuoles were accomplished, the vacuoles are
similar to those described by Couch (1975) and Hansen et al.
(1969). In these studies, juvenile and adult spot were ex-
posed to 5.0 ppb (parts per billion) Aroclor in flowing sea

Figure 8.   Liver (100X) Bouin's, H&E.   Exposed to 5.0 mg/l
            2,4-DNT for 56 days.   Parenchymal cell vacuolation
            probably the result of abnormal lipid accumulation.
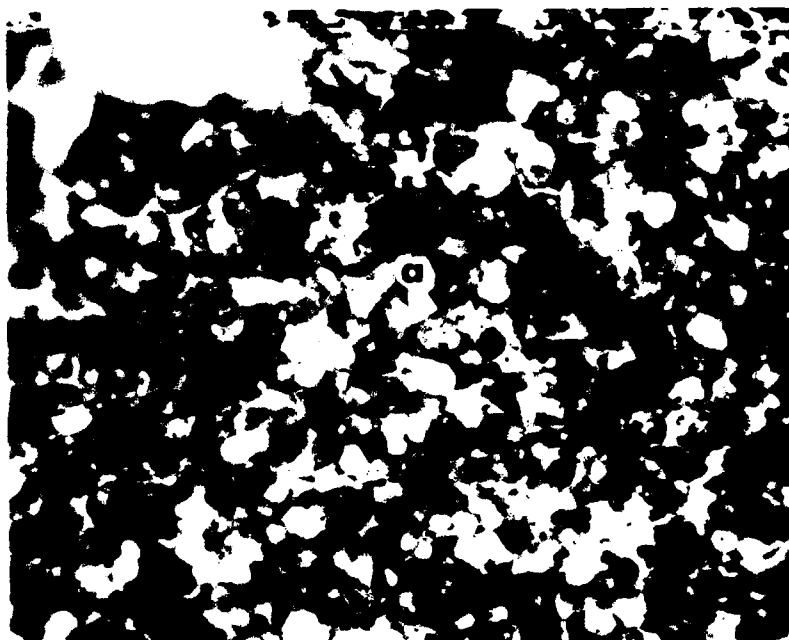
Figure 9.   Liver (1000X) Bouin's, H&E.   Exposed to 4.0 mg/l
            2,4-DNT for 49 days.   Parenchymmal cell vacuolation
            probably the result of abnormal lipid accumulation
            (a).

water for from 14-56 days. Fifteen to twenty micron smooth-edged vacuoles in hepatic cells indicated fatty accumulation in exposed spot. Couch (1975) reviewed the literature on the effects of pesticides (organochlorides and organophosphates) on fish hepatic tissue and noted that the most common non-specific liver lesion reported for fish after pesticide exposure was fatty change. In some cases (DDT, endrin, Aroclor 1254, Dursban) nutritional factors as well as pesticide exposure were suspected in the onset of fatty changes. The mechanism for such fatty chages is obscure. The liver seems the most likely site for 2,4-DNT and other compounds to act and cause fatty changes. Blocking, inhibition, or reducing the activity of some enzyme system essential in oxidation of fatty acids seems a possibility. For example, Norm and Bremer (1964) noted that high specific activity of palymityltransferase catalyzes the transfer of long-chain fatty acids. In cod and other fish, the specific activity of this enzme is highest in liver, heart and kidney (Jonas and Bilinski, 1964).

Figure 10 and Figure 11 demonstrate the most striking liver lesions found in the study. In the 8.0 mg/1 2,4-DNT exposure series, livers with necrotic foci in combination with edema/red blood cell infiltration were found in approximately 20 percent of the fish examined from day 49 to day 56. It is proposed that the necrotic foci are usually the result of extensive fatty deposits (fatty necrosis). In the 8 mg/1 2,4-DNT exposure series, lipid deposits are sometimes near necrotic foci. In similar studies using Aroclor, extensive vacuolation due to lipid accumulation was described as an intermediate pathological response. The final outcome was focal necrosis, sinusoidal congestion and extreme fatty changes (Couch, 1975).
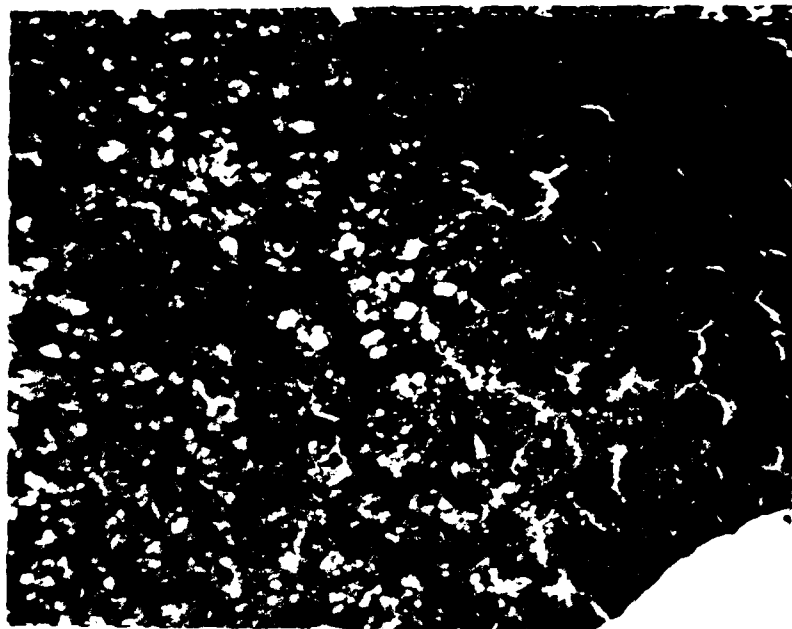
Figure 10.   Liver (400X) Bouin's, H&E.   Exposed to 8.0 mg/l
2,4-DNT for 49 days.   Foci of necrotic paren-
chyma (a) glycogen depletion (b).

Figure 11.   Liver (400X) Bouin's, H&E.   Exposed to 8.0 mg/l
2,4-DNT for 56 days.   Foci of necrotic parenchyma
(a) with edema/red blood cell infiltration (b).

Spleen

The bluegill spleen is a crescent shaped organ attached
to the portal vein. It is covered with a squamous epithelium
over a very thin fibrous connective tissue layer. Blood
sinuses are found beneath the connective tissue layer. The
organ (Figure 12) is also composed of red pulp and spenic
corpuscles with white pulp. Red and white pulp are clearly
visible and similar to channel catfish spleen (Grizzle and
Rogers, 1976). However, in rainbow trout red and white pulp
were less distinct (Anderson and Michum, 1974).

Figure 13 shows spleen with cytoplasmic inclusions in
bluegills exposed to 4.0 mg/l 2,4-DNT for 45 days. On day
45, these cytoplasmic inclusions were present in 40 percent
of the fish examined in the exposure group. It is known
that the degradation of hemoglobin within red blood cells
results in a golden brown pigment, hemosiderin, which accum-
ulates in irregularly granular masses in the cytoplasm of
phagocytes. There is normally some hemosiderin in the
phagocytes of spleens. However, in a disease or toxic situ-
ation which causes a more rapid destruction of red blood
cells, the amount of hemosiderin is markedly increased (Bloom
and Fawcett, 1975). Grover (1968) reported that bluegills
with lower condition factors had higher deposits of hemo-
siderin, which might be related to stress, in their spleens.
As previously discussed, the major physiological response
to 2,4-DNT in humans and other mammals is methemoglobinemia.
There may be some association between 2,4-DNT and increased
red blood cell destruction in fish resulting in large
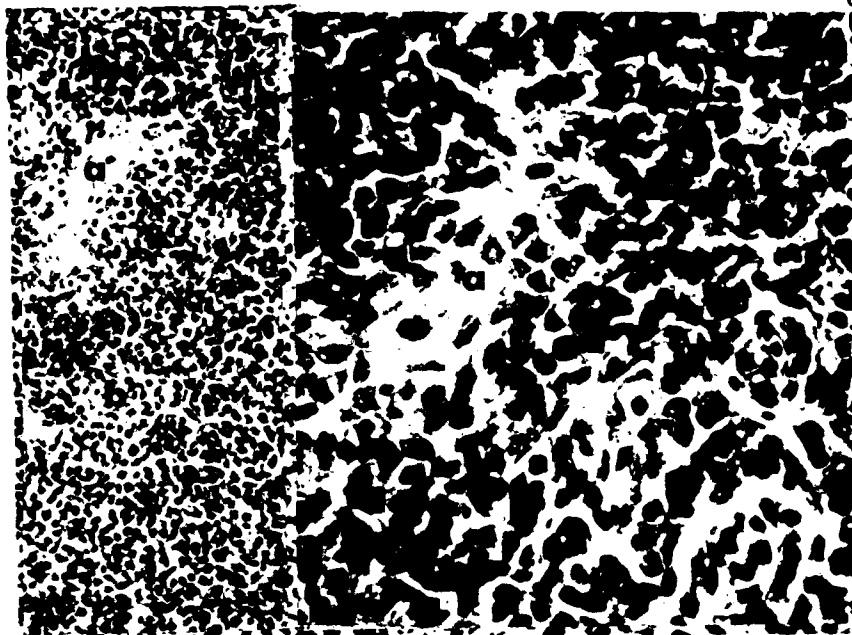
Figure 12.   (A) Spleen (400X) Bouin's, H&E.   Normal control
spleen, white pulp (a) and red pulp (b).
(B) Normal spleen (1000X) Bouin's, H&E. White
pulp (a) and red pulp (b).

Figuer 13.   Spleen (400X) Bouin's, H&E.   Exposed to 4.0 mg/l
2,4-DNT for 45 days.   Cytoplasmic inclusions,
probably hemosiderin.   Marked increase in hemo-
siderin accumulation from control spleens.

hemosiderin deposits in spleen tissue. However, the case for
this argument is not strong due to the large number of normal
spleens at other concentrations of 2,4-DNT tested.

Trunk Kidney

The kidney of the juvenile bluegill sunfish is position-
ed retroperitoneally along the body cavity. There are two
regions, the head and trunk kidney, that are morphologically
different. The organization of the bluegill sunfish kidney
is similar to that of the rainbow trout (Anderson and Mitchum,
1974) but dissimilar to the channel catfish. The kidney of
the channel catfish is fused bilaterally and divided into
completely separated anterior and posterior portions (Grizzle
and Rogers, 1976). In the juvenile bluegill sunfish, the
trunk kidney is composed of nephrons. Each nephron is com-
posed of a renal corpuscle and a renal tubule. There is a
little hemopoetic tissue scattered throughout the trunk kidney.
The renal corpuscle is composed of a (Figure 14) glomerulus
and surrounding Bowman's capsule. The glomerulus consists
of a mass of endothelial cells (Grizzle and Rogers, 1976)
which are separated from the viceral epithelium of Bowman's
capsule. A space surrounded the glomerulus and is surrounded
by the parietal epithelium of Bowman's capsule. It should
be noted that the parietal layer of Bowman's capsule is con-
siderably thicker than the viceral layer overlying the capil-
laries of the glomerulus. The renal tubules connect the
renal corpuscles to collecting ducts which empty into the
opistonephric ducts. In juvenile bluegill a proximal and
distal tubule can be identified. No clear first proximal or
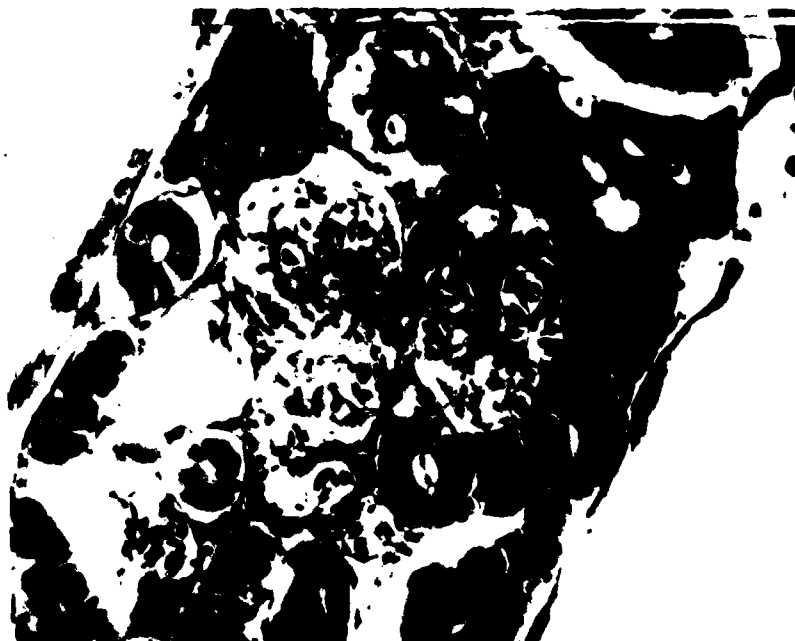
Figure 14.   Trunk kidney (400X) Bouin's, H&E.   Normal control
kidney.   Portions of the distal tubule (a)
proximal tubule (b) and renal corpuscle (c).
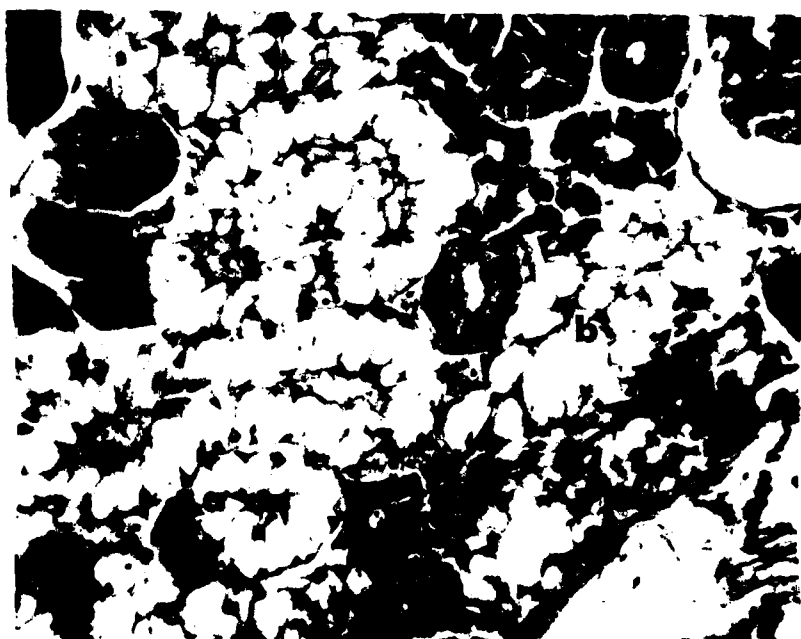
Figure 15.   Trunk kidney (400X) Bouin's, H&E.   Exposed to 5.0
mg/l 2,4-DNT for 56 days.   Markedly   atypical
tubule with basally displaced nuclei and constricted
lumen (a) and tubule necrosis (b).

second proximal segment separation can be found as for the
channel catfish (Grizzle and Rogers, 1976). The proximal seg-
ment in the bluegill nephron consists of columnar epithelium
with some nuclei centrally located while others are basilly
located. The distal tubules consist of low columnar to cuboi-
dal epithelial cells. The lumens of the distal tubules are
more constricted than the proximal tubules. Nuclei are more
basilly located in the epithelial distal tubule cells.

Atypical tubules and tubule necrosis in trunk kidney
were observed in 10 percent to 20 percent of the tissues
examined (Table 28) from day 49 to day 56 in the 5.0 mg/l and
8.0 mg/l 2,4-DNT exposures. Atypical tubules appear to be
the result of massive vacuoles in the cytoplasm of the epithe-
lial cells. Hentschel and Finkenstadt (1980) and Kendall
(1978) reviewed some typical artifacts in kidney tissue prep-
arations. The tubule lesions noted in the 2,4-DNT study
do not appear to be artifacts due to fixative or staining
procedures. Walsh and Ribelin (1975) in a review of the
pathology of pesticide poisoning noted that the epithelium
of the small distal portion of the convoluted tubule in lake
trout frequently contained large cytoplasmic vacuoles. They
were equally prominent in controls and fish exposed to DDT.
Significance of the vacuoles was unknown. In this study, the
atypical tubules shown in Figure 15 appear solely in the upper
exposure range of 2,4-DNT and were not observed in controls.
Necrosis of renal tubules has been observed in fish exposed
to mercury (Trump et al., 1975) and DDT (King, 1961; Mathur,
1965). In the author's opinion, the tubules exhibiting large

vacuoles with occluded tubules, represents a prenecrotic condition. Such tubules appear to be nonfunctional and would certainly reduce the efficiency of the trunk kidney in excretion and reabsorption of essential substances (Prosser, 1973). Necrosis is probably a dose response effect to the high concentration of 2,4-DNT tested. Some biochemical lesion probably precedes the necrosis. Evaluation of kidney tissue in the lower 2,4-DNT concentration exposures by electron microscopy might yield more information concerning the mechanisms resulting in atypical tubules and necrosis.

Lateral Line

In the juvenile bluegill sunfish, the lateral line extends down the side of the body to the base of the caudal fin. The cephalic portions extend over the head and the neuromasts are located in partially or completely enclosed canals. There are three canals in the head region similar to those described by Anderson and Mitchum (1974) for the rainbow trout, Grizzle and Rogers (1976) for the channel catfish, and Gardner and Yevich (1969, 1970) for Fundulus. They are the supraorbital, infraorbital and preopercular mandibular canal. Obstacle avoidance, location of prey and schooling orientation are some of the essential functions ascribed to this system (Anderson and Mitchum, 1974: Prosser, 1973). Figure 16 shows a longitudinal section of the lateral line of a juvenile bluegill. There is an accessory ossicle surrounding the canal. The neuromast has pear-shaped sensory cells with nuclei located in the upper portion of the neuromast sensory cells similar to neuromasts of the channel catfish (Grizzle and

Figure 16. (A) Lateral line (400X) Bouin's, H&E. Normal
            control lateral line. Neuromast with sensory cells
            (a), supporting cells with basally located nuclei
            (b) and stratified squamous epitherlium lining
            canal (c). (B) Lateral line (1000X) Control lateral
            line with neuromasts (a) and supporting cells (b).

Figure 17. (A) lateral line (100X) Bouin's, H&E. Exposed to 5.0
           mg/1 2,4-DNT for 56 days. Atypical neuromast cells
           (a), and necrotic epithelium (b). (B) Lateral line
           (400X). Atypical neuromast sensory cells (a)
           reduced dense nuclei (b).

Rogers 1976). The supporting cells (Figure 16) extend between
the sensory cells and have basally located nuclei. Squamous
stratified epithelium lines the canal. The lateral line
canal opens to the environment by regularly spaced pores. In
the head region, the ossicles are often fused in other bones
(Figure 17A).

Figure 17 shows the effect of 2,4-DNT on neuromasts
after 56 days exposure to 5.0 mg/l 2,4-DNT. The atypical
neuromast sensory cells were found in 20 percent of the tis-
sues examined (Table 28). Gardner (1975) in a review of the
literature noted severe cytoplasmic and nuclear degeneration
of sensory organ systems of some teleosts after exposure to
heavy metals (copper, silver, mercury) and the pesticide
methoxychlor, crude oil and pulpmill effluent. Mandibular
canal and neuromast lesions were observed in Fundulus exposed
to 0.5 mg/l copper, 0.5 mg/l mercury and 0.5 mg/l silver
(Gardner, 1975; Gardner and Yevich, 1969, 1970). The atypical
neuromast sensory cells and supporting cells were not similar
to those found by Gardner and Yevich (1969, 1970) however,
severe cytoplasmic change and dense nuclei clearly indicate
the neuromasts are not functional. This lesion could explain
ataxic or irregular swimming in bluegills observed at the
higher exposures to 2,4-DNT. In many cases death followed
highly irregular swimming activity. Lateral line lesions also
raises questions regarding the route of intoxication of 2,4-
DNT and other substances that produce similar lesions (Gardner
and LaRoche, 1973). The possibility of direct external
contact with 2,4-DNT in high concentrations causing fish

morbidity and mortality is proposed by the writer based on
the pathology noted in this study.

Respiratory System

In the juvenile bluegill sunfish, paired branchial
arteries from the ventral aorta carry deoxygenated blood to
four bronchial arches that extend from both sides of the pharynx.
Each gill consists of a gill arch with associated filaments
and lamellae (Figure 18). Cartillage is present to support
the gill arch and filaments. Stratified squamous epithelium
covers the filaments. The lamellae extend from both sides of
the filaments. Figure 19 shows nucleated blood cells within
the filament capillaries and the single layer of epithelial
cells surrounding the capillaries. The specialized cells
that support the lamellae are also demonstrated in Figure 19.
The basic histology of the normal bluegill respiratory system
is similar to that described for the channel catfish (Grizzle
and Rogers, 1976) and rainbow trout (Anderson and Mitchum
(1974).

Figure 20 and 21 demonstrate control gill filaments
and gill filaments exposed to 1.0 mg/l 2,4-DNT for 56 days,
respectively. Mild hyperplasia of interlamellar epithelium
is present in 10 to 20 percent of controls beginning 49 to 56
days. Ten to 40 percent of those fish exposed to 0.05 mg/l
to 2.0 mg/l 2,4-DNT for 35 to 56 days also develop mild
hyperplasia of interlamellar epithelium.

Figure 22 and Figure 23 demonstrate hypertrophy of
lamellar epithelium (2.0 mg/l 2,4-DNT, 42 days) and severe
hyperplasia of lamellar epithelium (5.0 mg/l 2,4-DNT, 49 days)

Figure 18. Control gill arch (100X) Bouin's, H&E. Gill arch
(a) paired afferent and efferent branchial arteries
(b) gill filament (c) (secondary) lamellae (d)
cartilage support (e).

Figure 19. Control lamellae (1000X) Bouin's, H&E. Single layer
of thin epithelial cells (a), nucleated blood
cells (b), supporting cells (c).
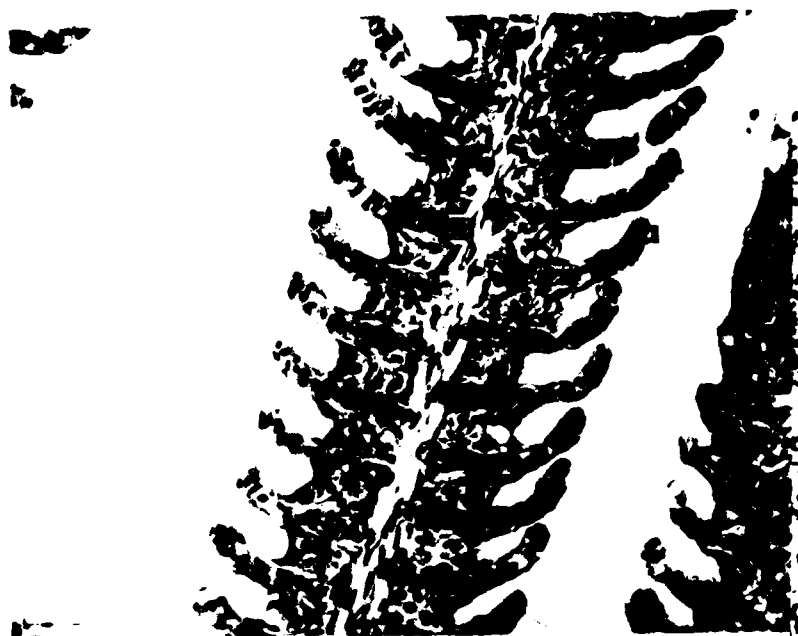
Figure 20. Gill (400X) Bouin's, H&E. Normal control gill. Longitudinal section of gill filament near the center of the filament where cartilage is absent. Lamellae (a) maximum length with well defined epithelial cells.

Figure 21. Gill (400X) Bouin's, H&E. Exposed to 1.0 mg/1 2,4-DNT for 56 days. Mild hyperplasia of inter-lamellar epithelium (a). Shortened lamellae due to angle of sectioning.
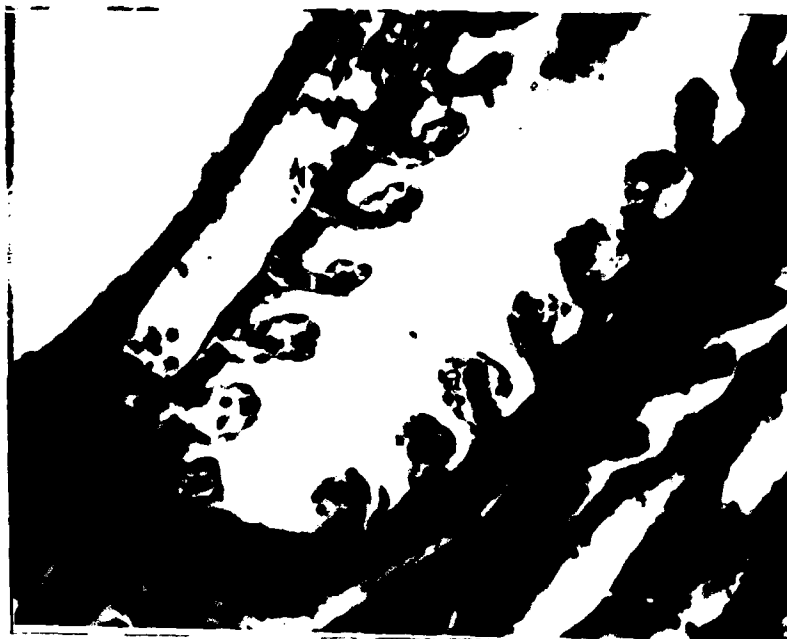
Figure 22. Gill (400X) Bouin's, H&E. Exposed to 2.0 mg/1 2,4-
DNT for 42 days.   Hypertrophy of lamellar epithe-
lium (edematous lamellae) (a).   Hypertrophied
epithelial cells occurred on the majority of gill
lamellae.

Figure 23. Gill (400X) Bouin's, H&E.   Exposed 5.0 mg/1 2,4-
DNT for 49 days. Severe hyperplasia of gill epithe-
lium (a). The epithelium is starting to proliferate
beyond the tips of the lamellae (b).

respectively. Hypertrophy of lamellar epithelium is found in the gills of bluegills after exposure to 0.5 mg/1-2.0 mg/1 2,4-DNT for 42 to 56 days. Severe hyperplasia of lamellar epithelium is demonstrated in gills from fish exposed to 5.0 mg/1 to 8.0 mg/1 2,4-DNT for 49 to 56 days.

Gill lesions such as hyperplasia and hypertrophy of gill epithelium within the lamellae indicate basic physiological problems (Eller, 1975). Moderate lamellar epithelial hyperplasia develops due to crowding and is frequently found in hatchery raised fish (Anderson and Mitchum, 1974). Burrows (1964) demonstrated that accumulated excretory products in hatchery grown salmonids cause gill epithelium hyperplasia and fused lamellae. Since the system used in this study was a flow through system, the author discounts the possibility of metabolic byproduct buildup as the cause of hyperplasia. Natural infections (bacterial or fungal) may account for a consistent low incidence of mild hyperplasia in control fish lamellae and the lamellae of fish exposed to the lower concentrations of 2,4-DNT (Eller, 1975). The mild hyperplasia observed in this study probably does not represent a severe physiological impairment of the bluegill respiratory system.

Hypertrophy of lamellar epithelium (edematous lamellae) due to pesticide exposure was reviewed by Eller (1975). Severe hypertrophy similar to that obtained in this study were found in fish exposed to sodium salt of 3-trifluoromethyl-4-nitrophenol (TFM) (Crisite and Battle, 1963), dichlorbenil (Cope, 1965) and sodium arsenite (Gilderhuis, 1966). The

hypertrophy was not uniform and involved limited areas of the gill lamellae. The type of hypertrophy due to 2,4-DNT exposure can result in significant loss of oxygen transfer efficiency. Several hyperplasia and proliferation of lamellar epithelial cells obviously reduce the surface area of the gills available for oxygen transfer. Similar severe hyperplasia of gill lamellae has been noted in pesticide exposures (Eller, 1975). Bluegills exposed to 3.0 mg/l diuron for 21 days produced severe hyperplasia similar to that produced in bluegills exposed to 5.0 mg/l to 8.0 mg/l 2,4-DNT (McCraren et al., 1969). Upon exposure to 2,4-DNT (5.0-8.0 mg/l) large areas of gill lamellae may be essentially nonfunctional. This severe response to high concentrations of 2,4-DNT represents a highly significant pathological reaction.

Other Organs/Tissues

No abnormalities were found in the digestive tract (gut), pancreas, integument, heart, gonad, head kidney and spinal cord. The normal histology of these bluegill tissues are found in the following appendices:

Appendix D-1   Digestive Tract (Gut)

Appendix D-2   Pancreas

Appendix D-3   Integument

Appendix D-4   Heart

Appendix D-5   Gonad

Appendix D-6   Head Kidney

Appendix D-7   Spinal Cord

Bioconcentration and Loss of 2,4-DNT in Bluegill Sunfish

Bluegills were exposed to 2.91±0.18 mg/1 2,4-DNT (C^{14} ring labeled 2,4-DNT plus unlabeled carrier) with an activity of 532±8 disintegrations per minute (dpm) per milliliter for 14 days. Aqueous radioactivity is detailed in Appendix E-1. Significant breakdown of 2,4-DNT in the exposure system did not occur as determined by gas chromatographic analyses of system water samples (Appendix E-1). The system water was only briefly exposed to low intensity light during daily fish feeding. The tanks were completely blacked out at all other times to reduce degradation by photolysis.

Exact sample weights for each tissue type were selected to reduce loss of counting efficiency due to color. Use of hydrogen peroxide to bleach the samples was effective in reducing color at the tissue sample weights selected. Conversion factors to compensate for reduction of counting efficiency were determined for each tissue sample weight selected and are detailed in Appendix E-2. Machine counting efficiency for C^{14} was determined with a prepared standard and was 96.62 percent efficient. The factor for machine efficiency is 1.035 to convert counts per minute (cpm) to dpm.

A control sample was prepared for each exposed group sample due to the extensive sample treatment and high LSC counting fluid. A single combined conversion factor (CCF) was calculated for each type of tissue to determine the bioconcentration factor (B.F.). The combined conversion factor was determined for each type of tissue and used in other calculations as follows.

B.F. = (exposed cpm - control cpm)(CCF)

$$CCF = \frac{\dfrac{1 \text{ gm}}{\text{sample (g)}} \left(\begin{array}{c}\text{Conversion factor}\\\text{for color}\end{array}\right) \left(\begin{array}{c}\text{Conversion factor}\\\text{for machine efficiency}\end{array}\right)}{532 \text{ dpm/ml of system water}}$$

The concentration of 2,4-DNT in each tissue sample was determined as follows.

$$\begin{array}{c}\mu\text{g 2,4-DNT}\\\text{per gm tissue}\end{array} = (B.F.) \left(\begin{array}{c}2.91 \mu\text{g per}\\\text{ml system water}\end{array}\right)$$

The data and above calculations for each type of tissue are provided in the following appendices:

Appendix E-3 Whole body

Appendix E-4 Brain

Appendix E-5 Kidney

Appendix E-6 Stomach/intestine

Appendix E-7 Gill

Appendix E-8 Liver-Pancreas

Appendix E-9 Striated muscle

The data was fitted to the zero and first order models previously described. The uptake and loss phase lasted for 3 to 4 days each. With such few data points, the uptake and loss phases fit a zero order model best. After uptake, the concentration of 2,4-DNT reached a plateau from which the maximum tissue level of 2,4-DNT was calculated by averaging plateau levels (usually day 4 to 14). More samples and thus data points may have resulted in a more gradual transition to a plateau or peak level and thus fit first order. However, considering that bioaccumulation is not significant from a food-chain standpoint and elimination is rapid, extensive

modeling of the data is not indicated.

The zero order uptake rates for 2,4-DNT in bluegill tissues are detailed in Table 30. The R-squared values indicate a good fit of the data to the zero order model. Bioconcentration factors (B.F.) and maximum concentrations of 2,4-DNT observed in each type of tissue are summarized in Table 31. Zero order loss rates for 2,4-DNT from bluegill tissues are listed in Table 32. High R-squared values indicate a good fit of the zero order model.

Table 30.

ZERO ORDER UPTAKE RATES* OF 2,4-DNT IN FISH TISSUE –
EXPOSED TO 3.0 mg/l 2,4-DNT[†] FOR TWO WEEKS

Tissue	Rate*± S.D.**	R-Squared Value (%)
Whole Body	15.36±1.40	93.0
Brain	43.76±9.52	77.1
Kidney	23.64±1.93	96.1
Stomach/Intestine	18.70±4.35	74.5
Gill	16.40±3.02	90.5
Liver	14.22±2.11	88.1
Striated muscle	6.02±0.586	95.5

*Rate = micrograms 2,4-DNT per gram of tissue per day uptake

**S.D. = Standard deviation

[†]C^{14} ring labeled 2,4-DNT (240 nanocuries/liter plus unlabeled carrier

Table 31.

MAXIMUM CONCENTRATION OF 2,4-DNT AND BIOCONCENTRATION
FACTOR IN FISH TISSUES - EXPOSED TO
3.0 mg/1 2,4-DNT[†] FOR TWO WEEKS

Tissue	Maximum 2,4-DNT Concentration in Tissue* ±S.D.**	Bioconcentration Factor***
Whole Body	72.19±9.57	24.8
Brain	199.37±12.94	102.8
Kidney	140.41±10.93	48.25
Stomach/intestine	84.98±9.79	29.20
Gill	68.74±12.41	23.62
Liver	85.61±8.02	29.41
Striated muscle	30.82±4.51	10.59

*Micrograms 2,4-DNT per gram tissue

**Standard deviation

***Concentration of 2,4-DNT in tissue ÷ concentration 2,4-DNT in the water

[†]C^{14} ring labeled 2,4-DNT (240 nanocuries per liter) plus unlabeled carrier

The maximum uptake rate (43.76 µg 2,4-DNT per gm tissue), B.F. (102.8) and loss rate (77.09 µg 2,4-DNT per gram tissue) occurred in brain tissue. A typical uptake and loss curve for brain tissue is shown in Figure 24. High brain lipid content and permeability of 2,4-DNT to the blood brain barrier may account for the rapid uptake and retention of 2,4-DNT. However, this does not account for the rapid elimination. Although no histological lesions were observed in fish brain upon exposure to 2,4-DNT, severe locomotor

Table 32.

ZERO ORDER LOSS (CLEARING) RATES* OF 2,4-DNT IN FISH
TISSUE – EXPOSED TO 3.0 mg/l 2,4-DNT[†] FOR TWO WEEKS

Tissue	Rate* ± S.D.**	R-Squared Value (%)
Whole Body	24.94±3.78	89.5
Brain	77.09±5.07	98.3
Kidney	31.30±4.73	89.6
Stomach/intestine	19.96±4.42	82.9
Gill	19.62±4.71	84.5
Liver	24.56±1.72	98.5
Striated muscle	9.53±1.43	91.6

*Rate = micrograms 2,4-DNT per gram tissue per day lost

**S.D. = Standard deviation

[†]C^{14} ring labeled 2,4-DNT (240 nanocuries per liter) plus unlabeled carrier.

impairment in fathead minnows (Hartley et al., 1977) and blue-gills (thisstudy) has been observed. Ellis et al. (1979) observed behavioral changes in dogs exposed to 2,4-DNT in which the most severe cases were accompanied by degnerative lesions in the cerebellum. Many of the symptoms of 2,4-DNT intoxication in humans previously discussed (McGee and McCausland, 1942; McGee et al., 1947) indicate the possibility of central nervous system effects.

The kidney was the site of second highest 2,4-DNT uptake rate (23.64 µg 2,4-DNT per gram tissue), B.F. (48.25) and 2,4-DNT loss rate (89.6 µg, 2,4-DNT per gram tissue).
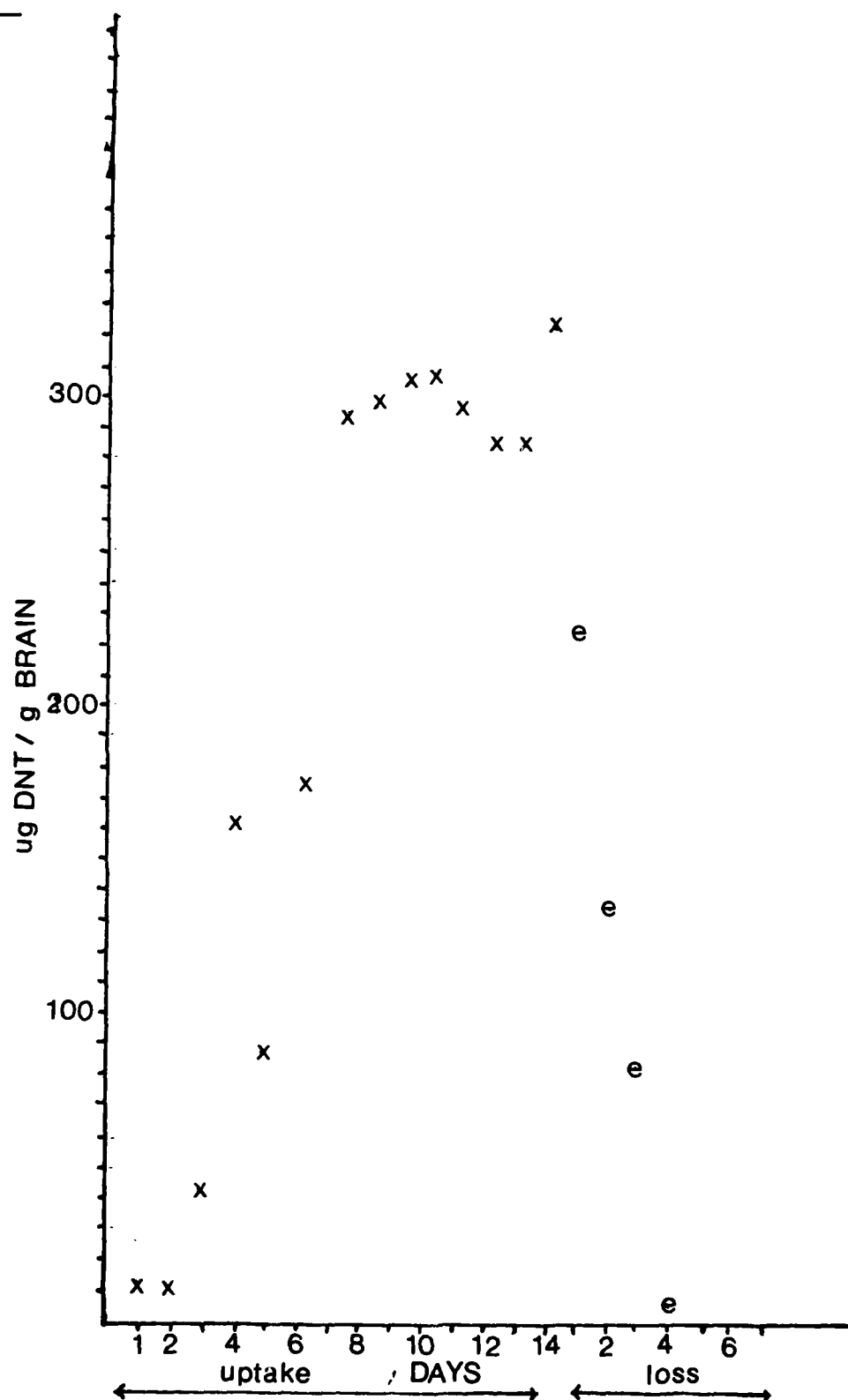
Figure 24.   Brain two week uptake and one week loss of 2,4-DNT.

This follows the pathological findings in this study where the most severe lesions were found in renal tubules. Ellis et al. (1979) found severe renal effects in mice including pathological cystic degeneration and a variety of tumors. The effects were more pronounced in male mice.

Uptake of 2,4-DNT was relatively slow in the liver (14.22 µg 2,4-DNT per gram tissue) and the B.F. (29.41) was approximately one half the B.F. of the kidney. The loss rate (24.56 µg 2,4-DNT per gram tissue) was third highest among the tissues tested. In this study, the liver was the site of extensive pathological response to 2,4-DNT as previously discussed. Ellis et al. (1979) found progressive development of hepatocellular carcinoma in rats exposed to 2,4-DNT. In humans, McGee et al. (1942) reported hepatic tenderness and jaundice in workers exposed to 2,4-DNT.

The third highest 2,4-DNT uptake rate occurred in the stomach and intestine (18.70 µg 2,4-DNT per gram tissue). The B.F. (29.2) and loss rate (19.96 µg 2,4-DNT per gram of tissue) were fourth highest of the tissues examined. No lesions of the stomach or intestine associated with 2,4-DNT exposure have been observed in fish, humans and other mammals. This agrees with the literature surveyed in which few intestinal or stomach lesions have been reported in teleosts. However, the higher 2,4-DNT uptake rate together with the lower B.F. indicates that the gut is a major route of entry for 2,4-DNT into the bluegill sunfish.

The fourth highest uptake rate of 2,4-DNT occurred in the gills (16.40 µg 2,4-DNT per gram tissue). However, the

B.F. (23.62) and loss rate (19.62 µg 2,4-DNT per gram tissue) were lowest except for striated muscle. Significant lesions were observed in bluegill respiratory tissue as previously discussed. The lesions observed may have in part been caused by contact toxicity. The uptake rate, low B.F. and loss rate of 2,4-DNT in the gills indicate that the gills are a major route of entry.

Striated muscle had the lowest 2,4-DNT uptake rate (6.02 µg 2,4-DNT per gram muscle), B.F. (10.59) and loss rate (9.53 µg 2,4-DNT per gram tissue). No lesions were observed in bluegill muscle. From the standpoint of the transfer of 2,4-DNT to humans via the food chain, fish striated muscle is the tissue of interest. Previous studies (SRI #31, 1978) reported a B.F. of 4 in bluegill muscle upon exposure to 1.0 mg/l 2,4-DNT. In this study, the higher driving force due to the 3.0 mg/l 2,4-DNT may have resulted in the higher striated muscle B.F. In both cases, the concentration of 2,4-DNT in muscle as well as other tissues was reduced to zero after three to four days in 2,4-DNT free water.

The whole body 2,4-DNT uptake rate (15.36 µg 2,4-DNT per gram tissue), B.F. (24.8) and loss rate (24.94 µg 2,4-DNT per gram tissue) reflects the combined response of the tissues examined plus those tissues not evaluated. For example, highly vascularized and lipid laden fish integument may also contribute to uptake and loss of 2,4-DNT. Action of 2,4-DNT on fish integument was evidenced by severe lateral line lesions in bluegills. It is important to note that in this study and others (Lee et al., 1975; Ellis et al., 1979

and SRI #31, 1978) 2,4-DNT is rapidly absorbed (24 to 96
hours) reaches relatively low bioconcentration levels, and
is rapidly eliminated (24-72 hours) in both fish and mammals.
However, 2,4-DNT is sufficiently toxic to produce histopath-
ological lesions at target organ sites.

CHAPTER 5

CONCLUSIONS

CONCLUSIONS

Baseline Bluegill Growth

Juvenile bluegill growth could be described by both the first order (exponential) model and the second order model. The use of the first order model is standard for fast growing fish (Ricker, 1958, 1971). First order growth constants at 21°C for juvenile bluegills were 0.0132-0.0144 day^{-1} and fall within the range for all age bluegills reported by Beitinger and Magnuson (1979) and Lemke (1977). Variation in this study was reduced by careful selection of uniform size (weight) at the onset of each growth series. Other growth models attempted, zero order and LOG-LOG models, were clearly inferior as growth descriptors.

Effects of 2,4-DNT on Growth

Two eight week exposure series of juvenile bluegill sunfish to sublethal concentrations of 2,4-DNT were conducted to determine the potential effect of this toxicant on tissues and growth. Since the solvent acetone was used as a carrier (concentration of acetone not to exceed 16.0 mg/l), a separate exposure series was conducted using 10.0 and 16.0 mg/l acetone. There were no significant effects on growth characteristics of the bluegill and no acetone associated histological lesions for 56 days.

In two 2,4-DNT exposure series, bluegills were exposed to 0.05, 0.5, 1.0, 2.0, 4.0, 5.0 and 8.0 mg/l 2,4-DNT for 56 days. Both the first and second growth constants indicated reduced growth rates as the concentration of 2,4-DNT

increased. The response was similar to that observed by
Pessah and Powles (1974) when pumpkinseed bluegills were ex-
posed to inhibitory temperatures. The first order growth
constants were significant at all concentrations of 2,4-DNT
tested when exposed groups were compared to the controls and
to each other (α=0.01). The threshold 2,4-DNT concentration
for subacute growth response in bluegills was approximately
0.05 mg/l 2,4-DNT. The upper limit that produces subacute
growth response without high mortality was greater than 4.0
mg/l and less than 5.0 mg/l 2,4-DNT. There was no bluegill
growth at 5.0 and 8.0 mg/l 2,4-DNT. Conclusions were the
same based on the second order model except that growth con-
stants (α=0.01) for the 0.05-0.5 mg/l 2,4-DNT and 0.5-1.00
mg/l 2,4-DNT pairs were not significantly different. The
data fitted the first and second order models equally well
from a statistical standpoint. Using the first order model
or second order growth model, the laboratory procedure used
qualifies as a bioassay or quantative toxicity test.

Baseline Histology

Baseline histological and growth characteristics of
the juvenile bluegill sunfish kept under laboratory conditions
were described. Although there are few complete histological
monographs of teleost fishes, the bluegill histology was
compared to extensive information on the channel catfish
(Grizzle and Rogers, 1976) and the trout (Anderson and Mitchum,
1974). In particular, the histology of bluegill liver, pan-
creas, spleen, kidney, lateral line system, respiratory system,
digestive tract (gut), integument, heart, and gonad was

described and documented in detail. In comparison with other
teleost fishes, the juvenile bluegill histology was similar
but with several unique variations particularly in the stomach
portion of the gut as detailed in Appendix D-1. However,
tissue structures in all cases were similar to the extent that
form and function did not appear significantly different from
other teleost fishes.

Histopathology of Bluegill Response to 2,4-DNT

During the 8 week exposure to 2,4-DNT, fish were exam-
ined weekly to determine the sites of histopathological
effects of 2,4-DNT and time of onset of the lesions. No ab-
normalities were found in the digestive tract (gut), pancreas,
integument, heart, gonad, head kidney and spinal cord. How-
ever, significant histological lesions were observed in
hepatic, spleen, trunk kidney, lateral line and gill tissues.
Ten to 30 percent of the control and exposed fish had glycogen
extracted livers indicative of high glycogen content but this
type of tissue response is not considered a pathological
condition (Couch, 1975). Ten to 40 percent of the exposed
and control fish had non-pathological mild gill hyperplasia.

Significant 2,4-DNT associated lesions were observed
with increasing frequency in fish exposed to 0.5 mg/l 2,4-DNT
or greater. Fish exposed to 0.5 mg/l and 2.0 mg/l 2,4-DNT
developed hypertrophy of gill lamellae (20%) at approximately
42-56 days exposure. Bluegills exposed to 4.0 mg/l 2,4-DNT
developed large hemosiderin inclusions in the spleen (40%)
at approximately 45 days exposure. Some fish exposed to
5.0 mg/l and 8.0 mg/l 2,4-DNT developed lipid accumulation

with associated necrotic foci in the liver (10-20%) after
49-56 days. They also developed atypical trunk renal tubules
(10-20%) and tubule necrosis (10-20%) after 49 days. Atypical
neuromast cells and necrotic epithelium of the lateral line
developed in 20 percent of the fish exposed to 5.0 mg/l 2,4-
DNT for 56 days.

Uptake and Loss of 2,4-DNT in Bluegill Tissue

Studies to determine the target organ systems or site
of action of 2,4-DNT were accomplished in two week C^{14} (ring
labeled) 2,4-DNT uptake studies with one week in 2,4-DNT-free
water to determine clearing. The 2,4-DNT did not bioaccumu-
late to any significant extent from the standpoint of food-
chain transfer. Target organ systems, however, were indicated
by high uptake rates and relatively high bioconcentration in
the brain, kidney, liver, stomach/intestine, and gills. As
previously noted, the most severe lesions occurred in the
kidney, liver and gills. Lateral line mechanoreceptor lesions,
high brain uptake and bioconcentration may account for the
frequent ataxis observed in fish exposed to high 2,4-DNT
concentrations (Hartley et al., 1977). Uptake rates of 2,4-
DNT in the gill and stomach/intestine indicate that they were
the major routes of entry. In this study and others (Lee et al.,
1975; Ellis et al., 1979 and SRI #31, 1978), 2,4-DNT was
rapidly absorbed (24-96 hours) reached relatively low bio-
concentration levels and was rapidly eliminated (24-72 hours)
when animals were placed in a 2,4-DNT free environment.

Use in Biomonitoring Systems

The use of the short-term procedures (8 weeks or less)

evaluated in this research as a means of evaluating toxic effects in the environment or as part of a biomonitoring system is restricted due to the professional expertise required and initial equipment investment needed.

The detailed growth studies as developed in this study confirm the first or second order model as adequate descriptors of juvenile fish growth. Such procedures for evaluating fish growth could be simplified by weighing fish twice; once at the beginning of the study and 8 weeks later. Growth constants could be developed based on the first or second order model described in this study and manpower requirements would be minimal. Weighing fish weekly and the introduction of anesthetic use increases manpower requirements as well as the technical skills required to accomplish the tasks. Biomonitoring systems that have available the resources of a histology laboratory could ship fixed tissues for preparation and mounting. An individual with the required professional skills could then review the slides and provide input to the toxicity evaluation process. Acute toxicity data, short-term subacute growth results and histopathological analyses might provide enough information to reduce the requirement for more extensive and costly life-cycle studies.

APPENDIX A

METHODOLOGY MATERIALS

APPENDIX A-1

ACTIVATED CARBON ADSORPTION OF 2,4-DNT

The bioassay system generates approximately 0.5 gpm of 0.7 mg/1 2,4-DNT or less. This influent was reduced to a maximum of 0.07 mg/1 2,4-DNT by the activated carbon treatment system.

The design of the treatment system was based on the following formula (Bohart-Adams):

$$t = \frac{No}{CoVo} \left[D - \frac{V}{KNo} \ln\left(\frac{Co}{C_B} - 1\right) \right]$$

where:

t = service time (hours)

v = linear flowrate (ft/hr)

D = depth of carbon bed (ft)

Do = critical depth of carbon bed (ft)

K = rate constant (ft^3/lb/hr)

No = adsorptive capacity (lb/ft^3)

Co = influent solute concentration (mg/1)

C_B = allowable effluent solute concentration (mg/1)

where:

$$\text{Slope} = \frac{No}{CoV} \qquad y \text{ intercept} = \ln\left[\frac{\frac{Co}{C_B} - 1}{K \quad Co}\right]$$

Subsequently the Do value for each application/loading rate is determined by the following:

$$Do = \frac{V}{KNo} \ln\left(\frac{Co}{C_B} - 1\right)$$

Table I shows the values for the two application/loading rates.

TABLE I

VALUES FOR THE COEFFICIENTS No, K, Do (Co = 0.7 mg/1 2,4-DNT)

Loading Rate gpm/ft^2	K ft^3/lb-hr	No lb/ft^3	Do inches
5	10,079.0	.08	1.3
10	16,798.8	.07	1.8

The values of K, No and Do are plotted as a function of loading rate of 2,4-DNT in Figure 1. The lines are drawn through two points because the lower loading rate (2.5 gpm/ft^2) used in the study did not result in break-through within the 24 hr. test period. Therefore, the design of the system was in the area of the graph where the raw data points exist.

The specifications for the system were as follows:

Flow - 0.5 gpm 2,4-DNT (Co = 0.7 mg/1 DNT)

Diameter of circular column 4.0 inches

Depth of column 3 ft.

Loading $\dfrac{.5 \text{ gpm}}{\pi r^2} = \dfrac{.5 \text{ gpm}}{\pi(.1666)^2} = 5.73$ gpm/ft^2

From Figure 1:

K = 11,100 ft^3/lb-hr

No = 0.08 lb/ft

$v = \dfrac{5.73 \text{ gpm}}{\text{ft}^2} \cdot \dfrac{60 \text{ min}}{\text{hr}} \cdot \dfrac{\text{ft}^3}{7.48 \text{ gal}} = 45.96$ ft/hr
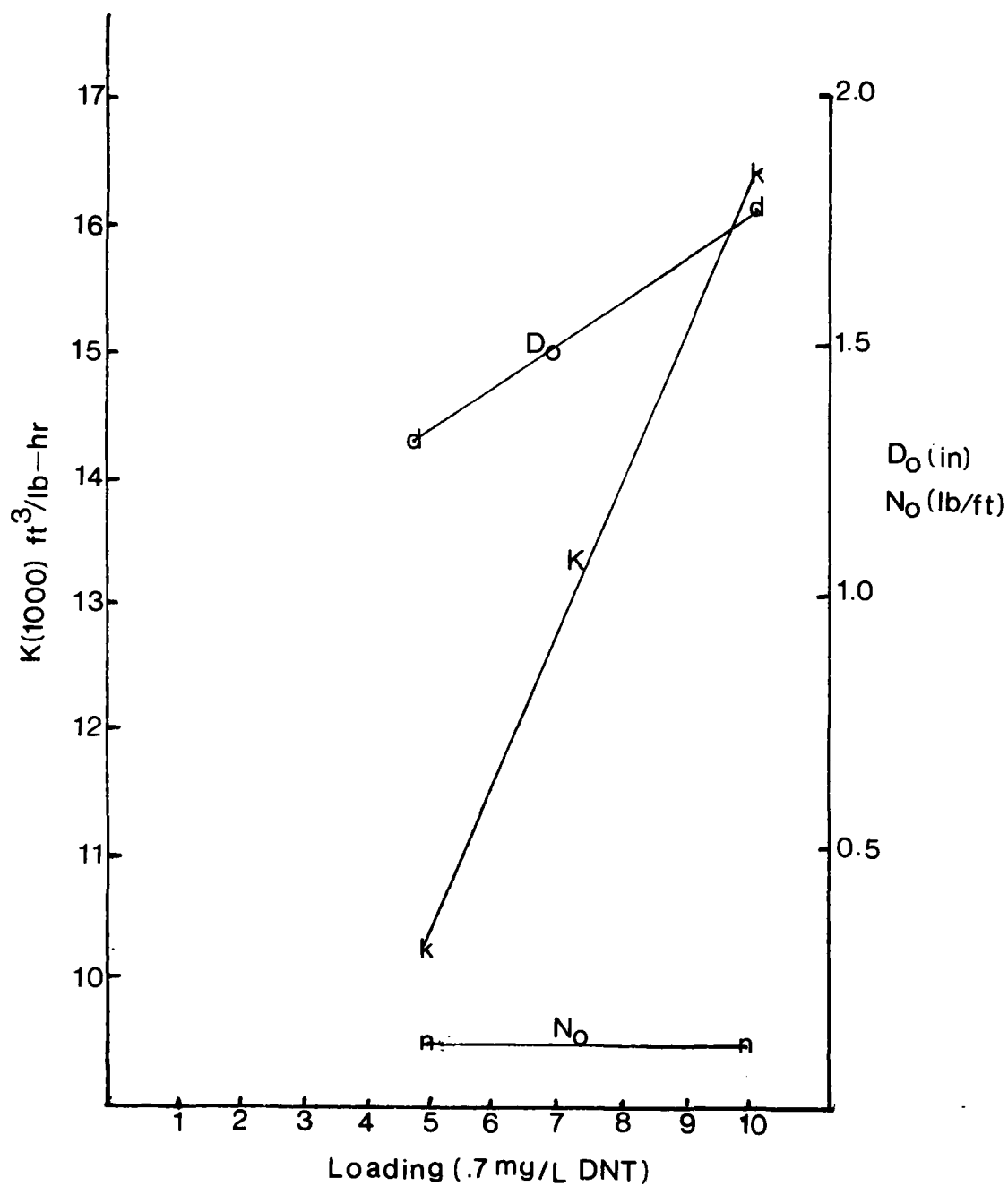
Figure 1. Kn, No, Do vs Loading Rate (gpm/ft^2) for 2,4-DNT

The service time (t) is now determined.

$$t = \frac{No}{CoV} \left[D - \frac{V}{KNo} \ln \left(\frac{Co}{C_B} - 1 \right) \right]$$

$$t = \frac{.08 \text{ lb}}{\frac{ft}{1} \frac{.0000436 \text{ lb}}{ft^3} \cdot \frac{45.96 \text{ ft}}{hr}} \left[3 \text{ ft} - \frac{45.96 \text{ ft}}{\frac{hr}{1} \frac{11100 \text{ ft}^3}{lb-hr} \frac{.08 \text{ lb}}{ft^3}} \ln \left(\frac{7}{.07} - 1 \right) \right]$$

$$t = \frac{.08 \text{ lb}}{\frac{ft}{1} \frac{.0000436 \text{ lb}}{ft^3} \frac{45.96 \text{ ft}}{hr}} \left[3 \text{ ft} - \frac{45.96 \text{ ft}}{\frac{88 \text{ hr ft}^3 \text{ lb}}{lb - hr \text{ ft}^3}} (2.1972) \right]$$

$$t = \frac{.08}{.002/hr} (3 - .1137)$$

$$t = \frac{.08}{.002/hr} (2.886)$$

$$t = 39.923 \text{ hr} (2.886)$$

$$t = 115.217 \text{ hrs}$$

$$\underline{t = 4.8 \text{ days}}$$

APPENDIX A-2

SUBLETHAL EFFECTS -- SELECTED TISSUES (SKIN, LATERAL LINE)

Author	Fish	Toxicant	Subacute Effect
Lateral Line/Skin			
LaRoche (1972)	mummichog	copper	lateral line lesions, pyknotic nuclei in squamous epithelium, germinating layer of epithelium vacuolated, abnormal appearing neuromast nuclei, olfactory chemoreceptors abnormal with hyperplasia of supporting epithelium
Gardner and LaRoche (1973)	mummichog atlantic silverside	copper	cellular changes in mechanoreceptors of the lateral line canal of the head, lesions of olfactory organs including chemoreceptive sites
Skin			
Davies et al.(1976)	rainbow trout	Lead	black tail, caudal fin erosion
McKone and Young (1971)	goldfish	mercury	excess mucus secretion
Cardwell et al. (1976)	fathead brook trout channel catfish	selenium dioxide	external hemorrhaging

APPENDIX A-3

SUBACUTE RESPONSES IN FISH PANCREAS, LIVER AND INTESTINE

Author	Fish	Toxicant	Subacute Effect
Ellis et al. (1937)		selenium	degeneration of hepatic tissue
Crandall and Goodnight (1963)	guppy	lead, zinc pentachlor phenate	degeneration of hepatic tissue
Baker (1969)	winter flounder	copper	fatty deposits in the liver
Jackim et al. (1970)	mummichog	variety of metals	changes in acid and alkaline phosphatase, catalase, xanthine oxidase and ribonuclease
Kendall (1972)	catfish	methyl mer- curic chloride	necrosis of liver and pancreas
Jackim (1973)	winter flounder mummichog	lead lead mercury cadmium zinc, silver	66% decrease in Liver ALA-D 22% decrease in Liver ALA-D inhibits ALA-D increases ALA-D
Gardner and LaRoche	mummichog	copper	focal areas of necrosis
Narbonne et al. (1973)	carp	lead nitrate	high liver glycogen
Hinton et al. (1973)	channel catfish	mercury	foci of necrosis in liver tissue

APPENDIX A-3 (continued)

Author	Fish	Toxicant	Subacute Effect
Morgan et al. (1973)	white perch hog choker	Baltimore Harbor water	decreased catalase activity in liver
Hansen et al. (1974)	pinfish	Aeroclor 1016	severe vacuolation in the pancreatic exocrine tissue surrounding the portal vein
Fromm and Schiffman (1958)	large mouth bass	cadmium	epithelial cell lining was sloughed glucose uptake inhibited pyloric caeca necrosis
King (1962)	brown trout	DDT	submucosal vacuolization epithelial degeneration
Mathur (1962)	Asian species	DDT	loss of goblet cells
Gardner and Yevich (1970)	mummichog	cadmium	necrosis of tips of mucous folds sloughed epithelium
Eisler et al. (1972)	mummichog	NTA	lesion of ileum and rectum lesions with clear vacuolated cytoplasm in columnar mucosal epithelium

APPENDIX A-4

SUBACUTE RESPONSES IN FISH GILL

Author	Fish	Toxicant	Subacute Effect
Carpenter (1925) (1927)(1937)	<u>Leuciscus</u> <u>minnows</u>	metallic salts	coagulation of gill mucus film over gill filaments
Jones (1939)	stickback	19 metallic salts	gill secretions that cover gill filaments
Schweiger (1957)	carp brook trout rainbow trout	cadmium	"damage" to gill tissue
Lloyd (1960)	rainbow trout	zinc sulfate	swelling of the gill lamellae and cytological breakdown of epithelium in 2.5 hours/tissue hypoxia
Matthiessen and Brafield (1973)	stickback	zinc	active chloride cells appeared in secondary lamellae. Coalescing of secondary lamellar epithelium
Lloyd (1965)	several species	zinc, copper lead	swelling of epithelial cells and separate from pilaster cells of the lamallae and finally slough off
Brown et <u>al</u>. (1968)	rainbow trout	ABS and zinc	loss of mucus cells, fusing of secondary lamellae thickening of epithelial wall
Amend et <u>al</u>. (1969)	rainbow trout	ethyl mercury phosphate	breakdown of gill epithelium

APPENDIX A-4 (continued)

Author	Fish	Toxicant	Subacute Effect
Baker (1969)	winter flounder	copper	chloride cells took the place of lamellar mucous cells. Vacuolated epithelial cells
Skidmore (1970)	rainbow trout	zinc	gill epithelium damaged
Gardner and Yevich (1970)	mummichog	cadmium	necrosis of gill filaments and respiratory lamallae after 20 hours
Freeman and Everhart (1971)	rainbow trout	aluminum hydroxide	gill hyperplasia
Colin et al. (1971)	goldfish	mercury	excessive mucus secretion
Burton et al. (1972)	rainbow trout	zinc	cytological damage and tissue hypoxia
Freeman (1973)	rainbow trout	aluminum	gill hyperplasia
Olson and Fromm (1973)	rainbow trout	mercury	gill cartillage damage
Balinski and Jones (1973)	rainbow trout	cadmium copper	50% inhibition of lactate oxidation by gill fillaments
Gardner et al. (1976)	atlantic silverside	waste motor oil	vacuolation of gill tissue

APPENDIX A-5

SUBACUTE RESPONSES IN FISH KIDNEY AND THE INTERRENAL GLAND

Author	Fish	Toxicant	Subacute Effect
Ellis et al. (1937)		selenium	degeneration of renal tissue
Crandall and Goodnight (1963)	guppy	lead nitrate zinc sulfate	reduction of renal peritubular lumphoid tissue, dilation of renal tubules degeneration of tubular epithelial cells
Hill and Fromm (1968)	rainbow trout	stress	elevation of plasma cortisol
Baker (1969)	winter flounder	copper	necrosis of renal tissue, vacuolated cells
Gardner and Yevich (1970)	mummichog	cadmium	reduction of eosinophils in the head kidney tissue, histological changes after 12 hours
Miettinen et al. (1972)	pike trout	methyl mercury	severe degeneration
Kendall (1972)	catfish	methyl mercuric chloride	necrosis of renal tubular cells thickening of renal glomerular basement membrane
Eisler et al. (1972)	striped bass mummichog	sodium nitrilo-acetic acid (NTA)	necrosis of proximal kidney tubules
Gardner and LaRoche (1973)	mummichog	copper	necrosis
Hinton et al. (1973)	channel catfish	methyl mercury	tubular cells desquammating into lumen of renal tubles; Basement membranes of glomeruli thickened

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APPENDIX A-6

SUBACUTE RESPONSES IN GONAD TISSUE

Author	Fish	Toxicant	Subacute Effect
Crandall and Goodnight (1963)	guppy	lead nitrate zinc sulfate	retarded and aberrant gonad development
Eller (1971)	cutthroat	endrin	hyperplasia of germinal layer and involution of some ova
Eller (1969)	redear sunfish	hydrothol 191	development of ova like cells in testes
Stock and Cope (1969)	guppy	TEPA	testicular astrophy and hypospermia
Sangaland and O'Halloran (1972)(1973)	brook trout	cadmium	testicular injury
Sangaland and Freeman (1974)	brook trout	cadmium	spermatozoa destruction, enlarged blood vessels, lobules disorganized, lobule boundry cells necrosis

APPENDIX A-7
EXTRACTION OF 2,4-DNT FROM WATER

Solutions of 0.01 mg/l, 0.05 mg/l, 0.10 mg/l, 0.5 mg/l and 1.0 mg/l 2,4-DNT in water were prepared to evaluate extraction efficiency. A 500 milliliter sample was placed in a flask and sealed with a cork covered in foil. Next, 5 ml of hexane was added to the sample. The sample was shaken at high speed on a wrist-action mechanical shaker for six minutes. The sample was then placed in a separator funnel. The water was then drained back to the flask. Another 5 ml of hexane was added to the sample. The sample was shaken again for 15 minutes. The sample was placed in the separator funnel. The water layer was removed and the total volume of approximately 10 ml of hexane was made up to exactly 10 ml. The 5 microliters samples were then injected into the GC. The percent recovery of 2,4-DNT from the water samples was determined. They were as follows. The overall average extraction efficiency is 79.6%. Eighty percent was used in calculations in the study.

Sample mg/l 2,4-DNT	Trial 1 % Recovery	Trial 2 % Recovery
0.01	59.4	65.7
0.05	79.1	81.4
0.10	84.2	84.8
0.50	83.8	85.3
1.00	86.7	85.6
Average:	78.64%	80.56%

APPENDIX A-8

TOXICOLOGY OF ACETONE CARRIER

Acetone is a volatile, colorless liquid that is miscible with water. It is used as a solvent for many organic compounds. The oral LD_{50} for rats is 9.75 gm/kg of body weight (Henson, 1959).

In tap water with adequate oxygen, sunfish were killed by 14,250 mg/l acetone (Ellis, 1937; Shelford, 1917). The 48 hr and 96 hr LD_{50} for the mosquito fish (Gambusia affinis) was reported as 13,000 mg/l acetone (Wallen et al., 1957).

The threshhold concentrations for immobilization of organisms in Lake Erie water at $20^{o}-25^{o}C$ were reported by Anderson et al. (1948). Values ranged from 6,500-13,600 mg/l acetone for invertebrates and 14,250 mg/l acetone for fish.

APPENDIX B

ACETONE CARRIER STUDY

APPENDIX B-1

GROWTH DATA - WEIGHT OF FISH (GRAMS) FOR ACETONE CARRIER STUDY

CONTROL

Fish No.	Days											
	0	5	10	15	20	25	30	35	40	45	50	55
1	0.54	0.57	0.59	0.66	0.67	0.73	0.76	0.78	0.86	0.91	1.03	1.08
2	0.55	0.61	0.63	0.64	0.65	0.72	0.76	0.79	0.87	0.91	0.97	1.12
3	0.56	0.59	0.60	0.65	0.68	0.72	0.77	0.79	0.85	0.95	1.03	1.18
4	0.53	0.60	0.63	0.67	0.70	0.68	0.76	0.83	0.86	0.92	1.10	1.25
5	0.54	0.58	0.63	0.65	0.67	0.70	0.79	0.73	0.79	0.87	1.09	1.03
6	0.56	0.61	0.64	0.69	0.65	0.73	0.75	0.74	0.86	0.94	1.04	1.09
Mean	0.547	0.593	0.620	0.660	0.670	0.713	0.765	0.777	0.848	0.917	1.043	1.125
S.D.*	0.012	0.016	0.020	0.018	0.019	0.020	0.014	0.037	0.029	0.028	0.047	0.079

*Standard deviation

APPENDIX B-2

GROWTH DATA - WEIGHT OF FISH (GRAMS) FOR ACETONE CARRIER STUDY

10.0 mg/1 ACETONE

Fish No.	Days											
	0	5	10	15	20	25	30	35	40	45	50	55
1	0.52	0.55	0.58	0.64	0.66	0.71	0.75	0.79	0.85	0.86	1.11	1.11
2	0.51	0.54	0.63	0.64	0.66	0.74	0.74	0.75	0.88	0.92	0.99	1.08
3	0.53	0.56	0.60	0.63	0.68	0.73	0.72	0.80	0.87	0.97	1.10	1.09
4	0.53	0.56	0.57	0.66	0.68	0.67	0.76	0.79	0.84	0.90	1.11	1.18
5	0.55	0.58	0.60	0.65	0.66	0.71	0.76	0.78	0.77	0.90	1.13	1.07
6	0.54	0.58	0.61	0.67	0.67	0.74	0.73	0.75	0.80	0.96	1.07	1.12
Mean	0.530	0.562	0.598	0.648	0.668	0.717	0.743	0.777	0.835	0.918	1.085	1.108
S.D.*	0.014	0.061	0.021	0.015	0.010	0.027	0.016	0.022	0.042	0.041	0.051	0.040

*Standard deviation

APPENDIX B-3

GROWTH DATA - WEIGHT OF FISH (GRAMS) FOR ACETONE CARRIER STUDY

16.0 mg/l ACETONE

Fish No.	Days											
	0	5	10	15	20	25	30	35	40	45	50	55
1	0.48	0.57	0.60	0.65	0.68	0.73	0.70	0.74	0.77	0.91	1.09	1.08
2	0.50	0.55	0.59	0.61	.66	0.72	0.77	0.78	0.86	0.97	1.15	1.15
3	0.49	0.55	0.63	0.66	0.70	0.71	0.69	0.81	0.88	0.97	1.07	1.18
4	0.52	0.55	0.59	0.70	0.66	0.75	0.77	0.78	0.88	0.92	1.09	1.20
5	0.51	0.58	0.61	0.66	0.69	0.69	0.74	0.80	0.81	0.88	1.17	1.11
6	0.52	0.58	0.64	0.65	0.70	0.77	0.77	0.77	0.86	0.99	1.12	1.09
Mean	0.503	0.563	0.061	0.655	0.681	0.728	0.740	0.780	0.843	0.940	1.115	1.135
S.D.	0.016	0.015	0.021	0.029	0.018	0.029	0.037	0.024	0.044	0.043	0.039	0.049

*Standard deviation

APPENDIX B-4

GROWTH CONSTANTS, FIRST ORDER EQUATIONS AND
R-SQUARED VALUES FOR ACETONE CARRIER SCREENING SERIES

mg/l Acetone	First Order Equation	Constant a_1*	R-Squared
Control	WT = -0.618+0.0122 lnT	0.0122±0.0003	94.8%
10 mg/l	WT = -0.655+0.0131 lnT	0.0131±0.0003	95.6%
16 mg/l	WT = -0.667+0.0138 lnT	0.0138±0.0004	94.7%

*±Standard deviation.

APPENDIX B-5

TUKEY'S PAIRED COMPARISON PROCEDURE FOR FIRST ORDER
CONSTANTS IN THE ACETONE CARRIER GROWTH EFFECTS
SCREENING SERIES

α = 0.01 (error rate)

Confidence interval: ±0.0360

	ACETONE CONCENTRATION		
	Control	10.0 mg/l	16.0 mg/l
Mean First Order Constant	0.0122	0.0131	0.0138
		NS*(0.0009)**	NS(0.0016)
			NS(0.0007)

 *No significant difference between pairs of constants.
**Actual difference between pairs of constants.

APPENDIX B-6

HISTOPATHOLOGY RESULTS OF ACETONE SCREENING SERIES
ACETONE CONCENTRATION

Tissue Examined	Control	10.0 mg/l	16.0 mg/l
Gill	Mild hyperplasia 20%*(day 49)	Mild hyperplasia 20% (day 56)	Mild hyperplasia 40%**(day 49)
Liver	High glycogen	High glycogen 20%(day 49)	High glycogen 20%(day 49)
Lateral line	Normal	Normal	Normal
Kidney	Normal	Normal	Normal
Spleen	Normal	Normal	Normal
Gut	Normal	Normal	Normal
Heart	Normal	Normal	Normal
Gonad	Normal	Normal	Normal

*20 percent (1 out of 5 fish examined on day 49)
**40 percent (2 out of 5 fish examined on day 49)

APPENDIX C

GROWTH STUDY

APPENDIX C-1

2,4-DNT ANALYSES* FOR SERIES A EXPOSURES
(0.5-8.0 mg/l 2,4-DNT)

	Expected 2,4-DNT mg/l				
Week	0.5	2.0	5.0	8.0	Control
0	0.48	1.82	5.30	8.41	0.00
1	0.54	1.95	5.14	8.13	-
2	0.45	2.15	5.09	7.93	-
3	0.51	2.03	5.08	8.08	0.00
4	0.40	2.09	4.79	7.95	-
5	0.49	2.40	4.96	8.12	-
6	0.53	2.23	5.07	8.07	0.00
7	0.55	2.10	5.32	7.89	-
8	0.59	2.07	5.21	7.90	0.00
Mean	0.504	2.093	5.106	8.053	0.00
S.D.**	0.057	0.164	0.165	0.164	0.00

*Determined by Gas Chromatographic Analysis of Bioassay Water.

**Standard Deviation.

APPENDIX C-2

2,4-DNT ANALYSES* FOR SERIES B EXPOSURES
(0.05-4.0 mg/l 2,4-DNT)

	Expected 2,4-DNT mg/l					
Week	0.05	0.50	1.0	2.0	4.0	Control
0	0.041	0.46	1.20	2.42	3.96	0.00
1	0.055	0.47	1.09	2.17	3.51	-
2	0.061	0.52	1.08	2.01	4.51	-
3	0.053	0.51	0.96	2.00	4.14	0.00
4	0.060	0.45	0.97	2.11	4.03	-
5	0.048	0.53	1.01	1.93	4.04	-
6	0.051	0.50	1.13	1.89	4.11	0.00
7	0.045	0.48	1.10	1.96	4.09	-
8	0.052	0.51	1.06	2.07	3.98	0.00
Mean	0.052	0.492	1.070	2.06	4.04	0.00
S.D.**	0.007	0.028	0.077	0.161	0.275	0.00

*Determined by Gas Chromatographic Analysis of Bioassay Water.

**Standard Deviation.

APPENDIX C-3

DISSOLVED OXYGEN (D.O.) CONCENTRATION (mg/l) FOR
SERIES A EXPOSURES (0.5-8.0 mg/l 2,4-DNT)

Day	D.O	Day	D.O	Day	D.O
0	7.8	19	8.5	38	7.7
1	8.0	20	7.6	39	8.5
2	8.2	21	8.5	40	8.4
3	8.3	22	7.5	41	7.9
4	8.1	23	7.7	42	7.6
5	7.8	24	7.7	43	7.7
6	8.3	25	8.3	44	7.7
7	8.2	26	7.6	45	7.6
8	7.7	27	8.5	46	8.0
9	7.5	28	8.5	47	7.6
10	7.8	29	8.2	48	8.2
11	8.5	30	8.0	49	8.0
12	8.3	31	8.1	50	7.7
13	7.9	32	8.2	51	8.5
14	8.0	33	7.9	52	8.0
15	7.8	34	7.7	53	8.3
16	7.9	35	8.2	54	8.3
17	8.1	36	8.5	55	8.2
18	7.7	37	7.9	56	7.8

Mean	8.01
S.D.*	0.309

*Standard Deviation

APPENDIX C-4

DISSOLVED OXYGEN (D.O.) CONCENTRATION (mg/1) FOR
SERIES B EXPOSURES (.05-4.0 mg/1 2,4-DNT)

Day	D.O.	Day	D.O.	Day	D.O.
0	7.7	19	8.2	38	7.4
1	7.8	20	8.3	39	8.1
2	8.2	21	8.2	40	7.6
3	7.6	22	7.8	41	8.0
4	7.7	23	8.0	42	8.0
5	8.2	24	7.8	43	8.3
6	8.3	25	7.9	44	8.2
7	7.3	26	7.4	45	8.2
8	7.3	27	7.7	46	7.3
9	7.5	28	8.0	47	7.7
10	7.8	29	7.7	48	7.9
11	7.5	30	7.3	49	7.5
12	8.2	31	7.9	50	7.7
13	8.3	32	7.4	51	7.6
14	7.7	33	7.7	52	8.3
15	7.3	34	8.3	53	7.7
16	8.1	35	8.3	54	8.1
17	8.1	36	7.5	55	7.4
18	7.4	37	7.3	56	7.9

Mean	7.82
S.D.*	0.335

*Standard Deviation

APPENDIX C-5

pH (-LOG HYDROGEN ION CONCENTRATION) FOR
SERIES A EXPOSURES (0.5-8.0 mg/l 2,4-DNT)

Day	pH	Day	pH	Day	pH
0	7.8	19	7.5	38	7.8
1	7.3	20	7.6	39	7.8
2	7.7	21	7.8	40	7.8
3	7.7	22	7.8	41	7.5
4	7.5	23	7.5	42	7.3
5	7.7	24	7.7	43	7.7
6	7.4	25	7.6	44	7.6
7	7.7	26	7.7	45	7.5
8	7.3	27	7.4	46	7.8
9	7.8	28	7.5	47	7.4
10	7.7	29	7.7	48	7.5
11	7.5	30	7.6	49	7.3
12	7.3	31	7.5	50	7.7
13	7.6	32	7.3	51	7.8
14	7.4	33	7.4	52	7.6
15	7.8	34	7.5	53	7.4
16	7.4	45	7.3	54	7.8
17	7.7	36	7.7	55	7.8
18	7.3	37	7.6	56	7.4

Mean	7.58
S.D.*	0.175

*Standard Deviation

APPENDIX C-6

pH (-LOG HYDROGEN ION CONCENTRATION) FOR
SERIES B EXPOSURES (0.05-4.0 mg/1 2,4-DNT)

Day	pH	Day	pH	Day	pH
0	7.5	19	7.8	38	7.8
1	7.6	20	7.8	39	7.5
2	7.3	21	7.5	40	7.5
3	7.4	22	7.7	41	7.4
4	7.6	23	7.8	42	7.3
5	7.5	24	7.7	43	7.5
6	7.4	25	7.8	44	7.7
7	7.3	26	7.8	45	7.7
8	7.5	27	7.7	46	7.8
9	7.6	28	7.5	47	7.6
10	7.7	29	7.3	48	7.8
11	7.3	30	7.5	49	7.3
12	7.4	31	7.3	50	7.7
13	7.7	32	7.3	51	7.8
14	7.5	33	7.4	52	7.5
15	7.8	34	7.7	53	7.4
16	7.3	35	7.7	54	7.6
17	7.4	36	7.3	55	7.3
18	7.8	37	7.7	56	7.7

Mean	7.56
S.D.*	0.181

*Standard Deviation

APPENDIX C-7

TEMPERATURE ($^{\circ}$C) FOR SERIES A EXPOSURES
(0.5-8.0 mg/1 2,4-DNT)

Day	Temp	Day	Temp	Day	Temp
0	21.0	19	21.5	38	21.0
1	21.5	20	21.0	39	21.0
2	21.0	21	20.0	40	21.5
3	22.0	22	20.5	41	20.5
4	21.0	23	21.5	42	21.0
5	20.0	24	20.5	43	21.0
6	20.5	25	21.0	44	21.0
7	21.5	26	21.0	45	21.0
8	21.0	27	21.0	46	21.0
9	22.0	28	20.5	47	20.0
10	22.0	29	21.0	48	20.0
11	20.5	30	21.0	49	21.5
12	21.0	31	21.0	50	21.0
13	21.0	32	21.5	51	21.0
14	21.0	33	22.0	52	22.0
15	21.5	34	21.5	53	21.5
16	21.0	35	21.0	54	21.0
17	21.5	36	20.5	55	21.0
18	22.0	37	20.5	56	21.0

Mean	21.06
S.D.*	0.509

*Standard Deviation

APPENDIX C-8

TEMPERATURE (^{O}C) FOR SERIES B EXPOSURES
(0.05-4.0 mg/1 2,4-DNT)

Day	Temp	Day	Temp	Day	Temp
0	21.0	19	21.0	38	20.5
1	21.0	20	21.0	39	21.0
2	21.0	21	21.5	40	21.0
3	20.5	22	21.0	41	21.0
4	21.5	23	20.0	42	21.0
5	21.0	24	20.0	43	21.5
6	21.0	25	20.5	44	21.5
7	21.0	26	21.0	45	21.5
8	22.0	27	21.0	46	21.5
9	21.0	28	21.0	47	21.5
10	21.5	29	21.5	48	22.0
11	22.0	30	22.0	49	21.5
12	20.5	31	22.0	50	20.5
13	21.0	32	22.0	51	21.0
14	21.0	33	21.5	52	21.0
15	21.5	34	21.0	53	21.0
16	21.0	35	21.0	54	21.5
17	20.0	36	21.0	55	21.5
18	20.5	37	20.5	56	21.5

Mean	21.12
S.D.*	0.493

*Standard Deviation.

APPENDIX C-9

GROWTH DATA FOR SERIES A – WEIGHT IN GRAMS
0.5 mg/1 2,4-DNT

Fish No.	0	5	10	15	20	Days 25	30	35	40	45	50	55
1	0.49	*	*	*	*	*	*	*	*	*	*	*
2	0.50	0.54	0.58	0.60	0.57	0.72	0.69	0.70	0.75	0.88	0.96	1.00
3	0.50	0.60	0.58	0.64	0.66	0.68	0.70	0.74	0.82	0.89	0.96	1.15
4	0.51	0.55	0.55	0.61	0.65	0.68	0.73	0.84	0.83	0.83	0.90	0.98
5	0.50	0.55	0.57	0.68	0.68	0.65	0.79	0.76	0.88	0.90	0.99	0.92
6	0.52	0.50	0.50	0.62	0.68	0.60	0.74	0.76	0,82	0.88	0.93	0.97
Mean	0.503	0.548	0.556	0.630	0.648	0.666	0.730	0.760	0.820	0.876	0.948	1.004
S.D.**	0.010	0.036	0.336	0.032	0.046	0.045	0.039	0.051	0.046	0.027	0.034	0.087

*Fish death
**Standard deviation

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APPENDIX C-10

GROWTH DATA FOR SERIES A – WEIGHT IN GRAMS
2.0 mg/l 2,4-DNT

Fish No.	Days											
	0	5	10	15	20	25	30	35	40	45	50	55
1	0.50	0.51	*	*	*	*	*	*	*	*	*	*
2	0.51	0.53	0.51	0.51	0.55	0.58	*	*	*	*	*	*
3	0.50	0.49	0.54	0.53	0.52	0.57	0.59	0.62	0.66	0.70	0.70	0.83
4	0.52	0.51	0.50	0.53	0.54	0.55	0.61	0.64	0.66	0.74	0.75	0.80
5	0.49	0.51	0.50	0.52	0.54	0.56	0.61	0.62	0.64	0.72	0.78	0.86
6	0.50	0.53	0.53	0.55	0.56	0.56	0.58	0.61	0.68	0.69	0.76	0.78
Mean	0.503	0.513	0.516	0.528	0.542	0.564	0.598	0.623	0.660	0.713	0.748	0.818
S.D.**	0.010	0.015	0.018	0.015	0.015	0.011	0.015	0.013	0.016	0.022	0.034	0.035

*Fish death
**Standard deviation

APPENDIX C-11

GROWTH DATA FOR SERIES A - WEIGHT IN GRAMS
5.0 mg/l 2,4-DNT

Fish No.	0	5	10	15	20	Days 25	30	35	40	45	50	55
1	0.50	*	*	*	*	*	*	*	*	*	*	*
2	0.48	*	*	*	*	*	*	*	*	*	*	*
3	0.49	0.42	0.54	0.50	0.59	0.54	0.52	0.54	0.54	0.53	0.54	0.55
4	0.50	0.52	0.42	0.46	0.55	0.53	0.58	0.52	0.53	0.55	0.53	0.54
5	0.51	0.53	0.49	0.40	0.50	0.50	0.54	0.55	0.54	0.53	0.54	0.55
6	0.50	0.41	0.51	0.55	0.56	0.59	0.54	0.54	0.49	0.53	0.52	0.53
Mean	0.497	0.470	0.490	0.478	0.550	0.540	0.545	0.538	0.525	0.535	0.533	0.543
S.D.**	0.013	0.064	0.051	0.063	0.037	0.037	0.025	0.012	0.023	0.010	0.010	0.010

*Fish death
**Standard deviation

APPENDIX C-12

GROWTH DATA FOR SERIES A - WEIGHT IN GRAMS
8.0 mg/l 2,4-DNT

Fish No.						Days						
	0	5	10	15	20	25	30	35	40	45	50	55
1	0.50	*	*	*	*	*	*	*	*	*	*	*
2	0.50	*	*	*	*	*	*	*	*	*	*	*
3	0.51	0.49	0.50	*	*	*	*	*	*	*	*	*
4	0.50	0.49	0.52	0.50	0.49	0.48	0.48	0.49	0.49	0.49	0.52	0.52
5	0.48	0.48	0.52	0.51	0.48	0.49	0.48	0.49	0.48	0.49	0.53	0.53
6	0.50	0.50	0.51	0.52	0.52	0.53	0.54	0.53	0.53	0.53	0.51	0.54
Mean	0.498	0.490	0.513	0.510	0.497	0.500	0.500	0.503	0.500	0.503	0.520	0.530
S.D.**	0.010	0.008	0.010	0.010	0.021	0.027	0.035	0.023	0.027	0.023	0.010	0.010

*Fish death
**Standard deviation

APPENDIX C-13

GROWTH DATA FOR SERIES B - WEIGHT IN GRAMS
0.05 mg/1 2,4-DNT

Fish	Days								
No.	0	7	14	21	28	35	42	49	56
1	0.50	0.55	0.60	0.64	0.70	*	*	*	*
2	0.52	0.52	0.64	0.68	0.65	*	*	*	*
3	0.51	0.55	0.67	0.63	0.66	0.80	0.83	1.03	*
4	0.48	0.55	0.57	0.61	0.72	0.77	0.85	0.95	1.20
5	0.53	0.50	0.59	0.66	0.70	0.76	0.89	0.93	1.05
6	0.53	0.54	0.60	0.63	0.68	0.81	0.79	0.96	1.08
7	0.50	0.53	0.54	0.70	0.67	0.76	0.99	0.94	1.10
8	0.51	0.55	0.60	0.67	0.69	0.71	0.83	0.95	1.15
9	0.50	0.46	0.55	0.66	0.69	0.75	0.85	1.01	1.07
10	0.52	0.56	0.64	0.64	0.70	0.77	0.88	0.93	1.09
11	0.50	0.61	0.55	0.68	0.75	0.69	0.84	0.99	1.13
12	0.54	0.56	0.63	0.58	0.74	0.78	0.87	0.95	1.08
13	0.48	0.58	0.60	0.68	0.71	0.76	0.80	0.93	0.99
14	0.48	0.54	0.61	0.67	0.72	0.76	0.79	0.90	1.23
15	0.47	0.52	0.60	0.65	0.73	0.76	0.82	0.94	0.92
16	0.45	0.60	0.61	0.59	0.71	0.75	0.83	0.89	1.10
17	0.50	0.45	0.58	0.67	0.70	0.78	0.87	0.97	1.09
18	0.54	0.59	0.59	0.62	0.72	0.76	0.80	0.95	1.09
19	0.55	0.56	0.60	0.57	0.68	0.81	0.85	0.95	1.12
20	0.50	0.61	0.64	0.64	0.70	0.76	0.83	0.90	0.99
21	0.50	0.55	0.62	0.71	0.69	0.76	0.79	0.96	1.18
22	0.51	0.56	0.58	0.63	0.75	0.71	0.83	0.99	1.10
23	0.50	0.63	0.63	0.65	0.70	0.78	0.85	0.95	1.14
24	0.52	0.54	0.63	0.59	0.73	0.75	0.87	1.00	1.09
25	0.47	0.53	0.61	0.66	0.71	0.76	0.80	0.99	1.10
Mean	0.504	0.550	0.603	0.644	0.704	0.761	0.841	0.955	1.095
S.D.**	0.024	0.042	0.031	0.037	0.026	0.029	0.044	0.035	0.069

*Fish death
**Standard deviation

APPENDIX C-15

GROWTH DATA FOR SERIES B - WEIGHT IN GRAMS
1.0 mg/l 2,4-DNT

Fish	Days								
No.	0	7	14	21	28	35	42	49	56
1	0.50	*	*	*	*	*	*	*	*
2	0.48	*	*	*	*	*	*	*	*
3	0.53	0.49	0.58	0.61	0.70	0.67	0.85	*	*
4	0.49	0.53	0.52	0.54	0.67	0.75	0.85	0.86	1.00
5	0.51	0.47	0.52	0.58	0.67	0.76	0.74	0.95	1.01
6	0.47	0.54	0.58	0.59	0.66	0.71	0.77	0.89	1.01
7	0.51	0.51	0.55	0.60	0.66	0.66	0.77	0.91	0.94
8	0.52	0.53	0.53	0.55	0.63	0.71	0.81	0.91	0.98
9	0.47	0.53	0.53	0.61	0.66	0.76	0.80	0.90	0.99
10	0.45	0.55	0.57	0.60	0.59	0.75	0.76	0.88	0.92
11	0.50	0.55	0.45	0.61	0.54	0.75	0.86	0.87	0.90
12	0.54	0.49	0.57	0.50	0.60	0.69	0.79	0.87	0.92
13	0.48	0.52	0.54	0.55	0.61	0.73	0.74	0.85	0.93
14	0.49	0.51	0.55	0.55	0.65	0.69	0.76	0.86	0.97
15	0.50	0.55	0.55	0.56	0.59	0.69	0.76	0.86	0.95
16	0.49	0.54	0.54	0.57	0.62	0.67	0.75	0.85	0.96
17	0.49	0.51	0.56	0.58	0.62	0.68	0.75	0.84	0.95
18	0.50	0.52	0.51	0.57	0.64	0.67	0.80	0.87	0.94
19	0.51	0.55	0.49	0.55	0.66	0.64	0.72	0.86	0.90
20	0.48	0.53	0.48	0.57	0.55	0.64	0.71	0.82	0.89
21	0.52	0.47	0.53	0.59	0.56	0.65	0.69	0.74	0.91
22	0.50	0.55	0.47	0.58	0.63	0.66	0.71	0.79	1.02
23	0.52	0.47	0.48	0.51	0.55	0.66	0.70	0.81	0.98
24	0.47	0.55	0.57	0.49	0.61	0.64	0.73	0.80	0.95
25	0.50	0.46	0.55	0.60	0.56	0.73	0.72	0.82	0.97
Mean	0.497	0.518	0.531	0.568	0.619	0.694	0.763	0.855	0.954
S.D.**	0.021	0.030	0.037	0.034	0.045	0.039	0.048	0.046	0.039

*Fish death
**Standard deviation

GROWTH DATA FOR SERIES B - WEIGHT IN GRAMS
2.0 mg/l 2,4-DNT

Fish No.	Days								
	0	7	14	21	28	35	42	49	56
1	0.50	*	*	*	*	*	*	*	*
2	0.49	*	*	*	*	*	*	*	*
3	0.49	*	*	*	*	*	*	*	*
4	0.50	*	*	*	*	*	*	*	*
5	0.48	0.48	0.49	0.52	0.56	0.63	0.68	0.79	0.81
6	0.49	0.52	0.45	0.55	0.56	0.62	0.65	0.74	0.81
7	0.53	0.49	0.45	0.55	0.58	0.60	0.68	0.71	0.81
8	0.47	0.47	0.47	0.54	0.58	0.62	0.67	0.74	0.81
9	0.50	0.53	0.50	0.53	0.58	0.62	0.66	0.78	0.78
10	0.50	0.52	0.56	0.52	0.59	0.62	0.75	0.73	0.80
11	0.51	0.47	0.51	0.55	0.59	0.62	0.67	0.74	0.81
12	0.50	0.50	0.52	0.55	0.55	0.63	0.71	0.74	0.86
13	0.48	0.51	0.52	0.55	0.54	0.63	0.68	0.74	0.85
14	0.52	0.51	0.52	0.57	0.58	0.63	0.72	0.73	0.81
15	0.50	0.51	0.52	0.56	0.58	0.61	0.69	0.75	0.82
16	0.49	0.50	0.52	0.56	0.62	0.64	0.68	0.73	0.80
17	0.50	0.51	0.56	0.56	0.62	0.62	0.68	0.74	0.79
18	0.49	0.50	0.53	0.54	0.60	0.64	0.69	0.75	0.80
19	0.50	0.53	0.55	0.54	0.57	0.65	0.65	0.70	0.81
20	0.51	0.52	0.49	0.50	0.57	0.63	0.65	0.77	0.81
21	0.51	0.53	0.55	0.55	0.58	0.59	0.70	0.75	0.78
22	0.50	0.53	0.54	0.58	0.58	0.62	0.68	0.69	0.76
23	0.53	0.52	0.54	0.58	0.58	0.62	0.73	0.69	0.83
24	0.53	0.54	0.55	0.59	0.60	0.59	0.61	0.68	0.79
25	0.54	0.49	0.52	0.55	0.60	0.71	0.65	0.72	0.84
Mean	0.502	0.509	0.517	0.550	0.581	0.626	0.680	0.734	0.808
S.D.**	0.017	0.020	0.033	0.021	0.020	0.024	0.031	0.029	0.023

*Fish death
**Standard deviation

APPENDIX C-17

GROWTH DATA FOR SERIES B - WEIGHT IN GRAMS
4.0 mg/l 2,4-DNT

Fish	Days								
No.	0	7	14	21	28	35	42	49	56
1	0.49	*	*	*	*	*	*	*	*
2	0.47	*	*	*	*	*	*	*	*
3	0.47	*	*	*	*	*	*	*	*
4	0.47	*	*	*	*	*	*	*	*
5	0.47	*	*	*	*	*	*	*	*
6	0.47	0.49	*	*	*	*	*	*	*
7	0.47	0.49	0.50	0.51	0.51	*	*	*	*
8	0.47	0.49	0.50	0.51	0.51	0.52	0.53	0.55	*
9	0.47	0.49	0.50	0.51	0.51	0.52	0.53	0.55	0.57
10	0.49	0.50	0.51	0.50	0.51	0.52	0.53	0.55	0.57
11	0.49	0.50	0.49	0.50	0.51	0.52	0.53	0.54	0.57
12	0.49	0.50	0.49	0.51	0.52	0.53	0.52	0.54	0.57
13	0.49	0.50	0.51	0.50	0.51	0.51	0.53	0.54	0.58
14	0.49	0.49	0.50	0.52	0.53	0.51	0.52	0.55	0.58
15	0.48	0.48	0.51	0.52	0.51	0.51	0.53	0.54	0.58
16	0.48	0.49	0.51	0.50	0.51	0.52	0.54	0.56	0.57
17	0.46	0.48	0.50	0.51	0.51	0.52	0.53	0.55	0.57
18	0.46	0.50	0.50	0.51	0.51	0.52	0.53	0.55	0.56
19	0.46	0.50	0.50	0.51	0.53	0.52	0.53	0.55	0.57
20	0.46	0.50	0.51	0.50	0.51	0.52	0.54	0.55	0.56
21	0.49	0.50	0.50	0.51	0.51	0.52	0.54	0.54	0.56
22	0.49	0.48	0.50	0.50	0.53	0.52	0.53	0.56	0.56
23	0.49	0.48	0.50	0.51	0.51	0.52	0.52	0.55	0.54
24	0.49	0.48	0.50	0.50	0.51	0.52	0.53	0.55	0.57
25	0.49	0.48	0.49	0.51	0.51	0.51	0.52	0.54	0.57
Mean	0.478	0.491	0.491	0.507	0.514	0.518	0.529	0.548	0.568
S.D.**	0.012	0.009	0.009	0.007	0.008	0.005	0.006	0.007	0.010

*Fish death
**Standard deviation

APPENDIX C-18

RELATIVE GROWTH RATES (h) X 100 FOR EXPOSURE SERIES A

Time Days	Concentration of 2,4-DNT (mg/l)				
	Control	0.50	2.00	5.00	8.00
0	0	0	0	0	0
5	11.72	8.95	1.99	-5.43	-1.61
10	4.54	1.46	0.58	4.25	4.69
15	8.19	13.31	2.23	-2.45	-0.58
20	0.77	2.86	2.65	15.06	-2.55
25	7.67	2.78	4.06	-1.82	0.60
30	6.55	9.61	6.03	0.93	0
35	3.21	4.11	4.18	-1.28	0.60
40	6.61	7.89	5.94	-2.42	-0.59
45	11.54	6.83	8.03	1.90	0.60
50	11.87	8.22	4.91	-3.74	3.38
55	11.00	5.91	9.36	1.88	1.92

APPENDIX C-19

CUMULATIVE RELATIVE GROWTH RATES (h) X 100 FOR EXPOSURE SERIES A

Time Days	Concentration of 2,4-DNT (mg/1)				
	Control	0.50	2.00	5.00	8.00
0	0	0	0	0	0
5	11.72	8.95	1.99	-5.43	-1.61
10	16.26	10.41	2.57	-1.18	3.08
15	24.45	23.72	4.80	-3.63	2.50
20	25.22	26.58	7.45	11.44	-0.05
25	32.89	29.36	11.51	9.61	0.55
30	39.44	38.97	17.54	10.54	0.55
35	42.65	43.08	21.72	9.26	1.15
40	49.26	50.97	27.66	6.84	0.56
45	60.80	57.80	35.69	8.74	1.16
50	72.67	66.02	40.60	5.00	4.54
55	83.67	91.93	49.96	6.88	6.46

APPENDIX C-20

RESULTS OF TUKEY'S PAIRED COMPARISON PROCEDURE FOR
CONSTANT (m) - SERIES A EXPOSURES
ZERO ORDER MODEL

$\alpha = 0.05$

Confidence interval m ± 0.0522

	Concentration (mg/l) 2,4-DNT				
	Control	0.50	2.00	5.00	8.00
mean(m)	1.02	0.89	0.53	0.11	0.04
		S*(0.13)**	S(0.49)	S(0.91)	S(0.98)
			S(0.36)	S(0.78)	S(0.85)
				S(0.42)	S(0.49)
					S(0.07)

$\alpha = 0.01$

Confidence interval m ± 0.0647

	Concentration mg/l 2,4-DNT				
	Control	0.50	2.00	5.00	8.00
mean(m)	1.02	0.89	0.53	0.11	0.04
		S(0.13)	S(0.49)	S(0.91)	S(0.98)
			S(0.36)	S(0.78)	S(0.85)
				S(0.42)	S(0.49)
					S(0.07)

 *Statistically significant difference between pairs
**Actual difference between pairs

APPENDIX C-21

RESULTS OF TUKEY'S PAIRED COMPARISON PROCEDURE FOR
CONSTANT (n) - SERIES A EXPOSURES
LOG-LOG MODEL

$\alpha = 0.05$

Confidence interval n ± 2.6645

	Concentration (mg/1) 2,4-DNT				
	Control	0.50	2.00	5.00	8.00
mean(n)	26.58	25.60	17.45	6.43	1.42
		NS**(0.98)†	S*(9.13)	S(20.15)	S(25.16)
				S(19.17)	S(24.18)
				S(11.02)	S(16.03)
					S(5.01)

$\alpha = 0.01$

Confidence interval n ± 3.3034

	Concentration (mg/1) 2,4-DNT				
	Control	0.50	2.00	5.00	8.00
mean (n)	26.58	25.60	17.45	6.43	1.42
		NS(0.98)	S(9.13)	S(20.15)	S(25.16)
			S(8.15)	S(19.17)	S(24.18)
				S(11.02)	S(16.03)
					S(5.01)

*Statistically significant difference between pairs
**Not statistically different between pairs
†Actual difference between pairs

APPENDIX C-22

RELATIVE GROWTH RATES (h) X 100 FOR EXPOSURE SERIES B

Time Days	Concentration of 2,4-DNT (mg/1)					
	Control	0.05	0.50	1.0	2.0	4.0
0	0	0	0	0	0	0
7	7.53	9.13	8.70	4.23	1.39	2.72
14	10.70	9.64	5.77	2.51	1.57	0.00
21	9.33	6.80	4.23	6.97	6.63	3.26
28	10.37	9.32	10.64	8.98	5.64	1.38
35	9.12	10.18	10.08	12.12	7.75	0.78
42	9.37	10.51	12.07	9.94	8.63	2.12
49	12.27	13.56	11.14	12.06	7.94	3.59
56	20.93	14.66	11.25	11.58	10.08	3.65

APPENDIX C-23

CUMULATIVE RELATIVE GROWTH RATES (Σh) X 100 FOR EXPOSURE SERIES B

Time Days	Concentration of 2,4-DNT (mg/1)					
	Control	0.05	0.50	1.0	2.0	4.0
0	0	0	0	0	0	0
7	7.53	9.13	8.70	4.23	1.39	2.72
14	18.23	18.77	14.47	6.74	2.96	2.72
21	27.56	25.57	18.70	13.71	9.59	5.98
28	37.93	34.89	29.34	22.69	15.23	7.36
35	47.05	42.99	39.42	34.81	22.98	8.14
42	56.42	53.50	51.49	44.75	31.61	10.26
49	68.69	67.06	62.63	56.81	39.55	13.85
56	89.62	81.72	73.88	68.39	49.63	17.50

APPENDIX C-24

RESULTS OF TUKEY'S PAIRED COMPARISON PROCEDURE FOR
CONSTANT (m) - SERIES B EXPOSURES
ZERO ORDER MODEL

$\alpha = 0.05$

Confidence interval m ± 0.0168

	Concentration (mg/1) 2,4-DNT					
	Control	0.05	0.50	1.00	2.00	4.00
mean(m)	1.10	0.99	0.88	0.81	0.54	0.14
Signifi-cant pairs matrix		S*(0.11)	S(0.22)	S(0.29)	S(0.56)	S(0.96)
			S(0.11)	S(0.18)	S(0.45)	S(0.85)
				S(0.07)	S(0.34)	S(0.74)
					S(0.27)	S(0.67)
						S(0.37)

$\alpha = 0.01$

Confidence interval m ± 0.0198

	Concentration (mg/1) 2,4-DNT					
	Control	0.05	0.50	1.00	2.00	4.00
mean(m)	1.10	0.99	0.88	0.81	0.54	0.14
Signifi-cant pairs matrix		S*(0.11)**	S(0.22)	S(0.29)	S(0.56)	S(0.96)
			S(0.11)	S(0.18)	S(0.45)	S(0.85)
				S(0.07)	S(0.34)	S(0.47)
					S(0.27)	S(0.67)
						S(0.37)

*Statistically significant difference between pairs
**Actual difference between pairs

APPENDIX C-25

RESULTS OF TUKEY'S PAIRED COMPARISON PROCEDURE FOR CONSTANT (n) – SERIES B EXPOSURES LOG-LOG MODEL

$\alpha = 0.05$

Confidence interval n ± 0.8386

	Control	0.05	0.50	1.00	2.00	4.00
		Concentration (mg/1) 2,4-DNT				
mean(n)	33.79	30.67	28.98	28.96	21.72	5.95
Significant pairs matrix		S*(3.12)†	S(4.81)	S(4.83)	S(12.07)	S(27.84)
			S(1.69)	S(1.71)	S(8.95)	S(24.72)
				NS**(0.02)	S(7.23)	S(23.00)
					S(7.89)	S(23.01)
						S(15.77)

$\alpha = 0.01$

Confidence interval n ± 0.9905

	Control	0.05	0.50	1.00	2.00	4.00
		Concentration (mg/1) 2,4-DNT				
mean(n)	33.79	30.67	28.98	28.96	21.72	5.95
Significant pairs matrix		S(3.12)	S(4.81)	S(4.83)	S(12.07)	S(27.84)
			S(1.69)	S(1.71)	S(8.95)	S(24.72)
				NS(0.02)	S(7.23)	S(23.10)
					S(7.89)	S(23.01)
						S(15.77)

*Statistically significant difference between pairs
**Not statistically different between pairs
†Actual difference between pairs

APPENDIX D

HISTOPATHOLOGY STUDY

APPENDIX D-1

HISTOLOGY OF THE BLUEGILL DIGESTIVE TRACT (GUT)

Buccal Cavity (Figures D-1-1 and D-1-2)

The oral cavity begins with the lips and ends just prior to the first gill slit. The lips consist of a thick convoluted epithelium. Posterior to the lips, the epithelium becomes thinner. The most distinctive structures in the lip epithelium are villiform teeth that are located in the maxillary bone. There are a few goblet cells (mucus secreting cells) on the lips. They become more numerous posterior to the lips. A thin lamina propria and a submucosa of areolar connective tissue are located below the epithelium. Taste buds are frequently found in the oral cavity. Other locations of taste buds include the external integument, pharynx, esophagus and gill arches. In general, the taste buds project above the epidermis. The three cell types present in the bluegill taste buds are receptor cells, supporting cells and basal cells. The same cell types are present in other species such as the channel catfish (Grizzle and Rogers, 1976) and rainbow trout (Anderson and Mitchum, 1974).

Pharynx (Figures D-1-3 and D-1-4)

The pharynx is bounded laterally by the gills. From a longitudinal view, the folds of the pharynx are wide and flat internally with a rounded and more raised appearance in the center. The opercular epithelium is contiguous with the pharynx epithelium. Very few taste buds are present in the pharynx epithelium. Pharyngeal teeth and goblet cells are

Figure D-1-1. Buccal Cavity (100X) Bouin's, H&E. (a) villi-
form teeth; (b) lamina propria; (c) mucus
secreting cells; (d) maxillary bone; (e) sub-
mucosa; (f) aductor muscle.

Figure D-1-2. Taste Bud (1000X) Formalin, H&E. (a) epidermis;
(b) nerve fibers; (c) receptor cells (basally
located nuclei with cell processes extending
toward the surface); (d) supporting cells;
(e) basal cells.

Figure D-1-3. Pharynx (40X) Bouin's H&E. (a) pharynx; (b) heart; (c) epithelium; (d) lamnia propria; (e) pharyngeal teeth; (f) opercular cavity epithelium; (g) sub-mucosa; (h) pharyngeal bone.

Figure D-1-4. Pharynx (400X) Formalin H&E. (a) pharyngeal tooth with dentine like material present; (b) mucus secreting cell; (c) modified epithelial cells; (d) lamnia propria.

numerous in the pharyngeal epithelium. The pharyngeal teeth rest on pharyngeal bone. The pharyngeal teeth are surrounded by modified epithelial cells. A thin lamnia propria and thick submucosa are located beneath the pharyngeal epithelium. The anterior portion of the phyarynx has numerous cardiform (pad formation) pharyngeal teeth. The posterior portion has few teeth or taste buds but numerous goblet cells and a thick epithelium. The bluegill pharynx is very similar to that of the channel catfish (Grizzle and Rogers, 1976) and rainbow trout (Anderson and Mitchum, 1974).

Esophagus (Figues D-1-5 and D-1-6)

The esophagus is bounded by the pharynx (anterior) and stomach (posterior). Most of the esophagus consists of longitudinal folds that permit expansion during swallowing. There are four primary layers. There is the mucosa consisting of epithelium and lamina propria of fibrous connective tissue. There is a visible muscularis mucosae in the juvenile bluegill as in the channel catfish (Grizzle and Rogers, 1976). The other layers are the submucosa which consists of fibrous connective tissue, the muscularis and the serosa (fibrous connective tissue and squamous epithelium. These basic layers are found in both the channel catfish (Grizzle and Rogers, 1976) and rainbow trout (Anderson and Mitchum, 1974). The caudal (posterior) portion of the esophagus consist of some columnar epithelium. The folds become more broad and flattened and numerous mucus secreting cells (goblet cells) displace the epithelial cells near the stomach. The same histological structure of the caudal region of the esophagus was noted in the rainbow trout by Anderson and Mitchum, 1974).
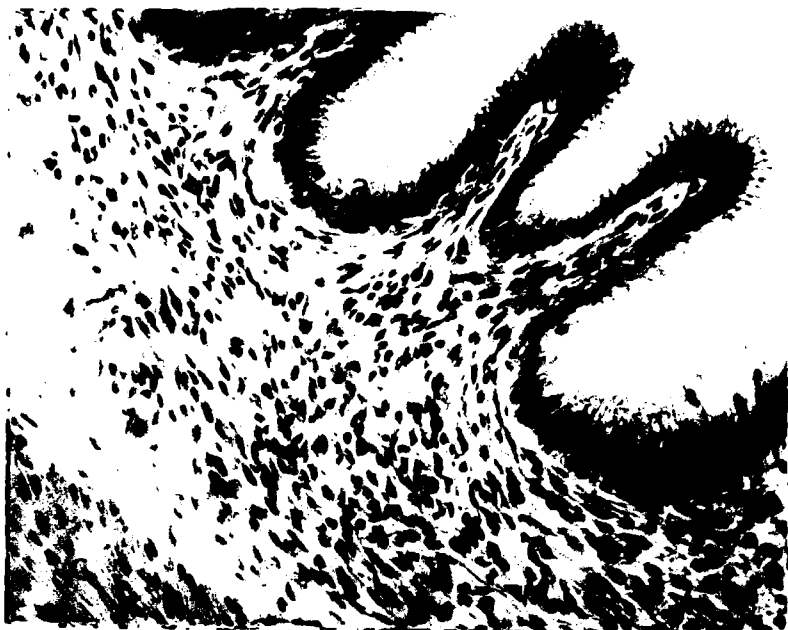
Figure D-1-5. Esophagus (400X) Bouin's H&E. (a) mucosa
(includes epithelium, lamina propria - fibrous
connective tissue); (b) submucosa (fibrous con-
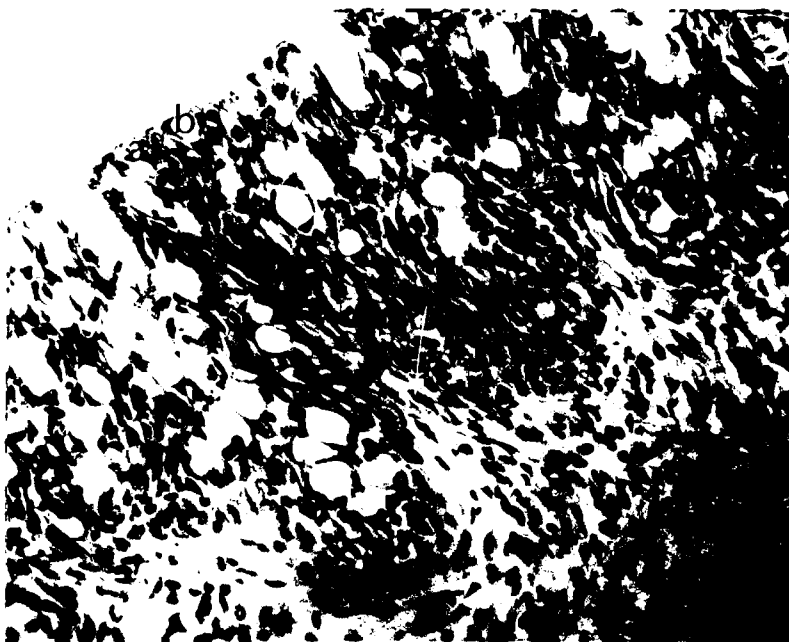nective tissue); (c) muscularis (muscle)

Figure D-1-6. Esophagus (posterior) (400X) Formalin H&E.
Posterior region of esophagus with (a) squamous
epithelium and numerous (b) goblet cells.

Stomach (Figures D-1-7 to D-1-12)

The stomach of the bluegill is J-shaped with two distinct regions. They will be referred to as the cardiac/ fundic region and the pyloric region. The histology of the juvenile bluegill stomach is varied and differs from the channel catfish (Grizzle and Rogers, 1976) and the rainbow trout (Anderson and Mitchum, 1974). The channel catfish stomach is divided into the fundic and pyloric region while the rainbow trout has a cardiac and pyloric region. In the bluegill stomach the anterior portion (cardiac/fundic region) consists of the transition zone (Figure D-1-7) from the esophagus in which there is a thin mucosa abundant in goblet cells, a reduced muscularis mucosae, submucosa, muscularis and serosa. However, the muscularis is composed of layers of smooth muscle. The serosa is mesothelium with reduced connective tissue.

Three other distinct areas occur in the cardiac/fundic region. In all three regions the submucosa, muscularis and serosa are as previously described for the transition zone. It is the mucosa that differs. The most anterior and the mucosa has broad-flat folds composed of simple columnar epithelium (Figure D-1-8) with basally located muscle. There are very few short tubular gastric glands that empty into pits and a few goblet cells are still present. Secondly, the mucosa becomes flat (Figure D-1-9) but retains the cell structure previously described. The third area of the cardiac/fundic region is typified by the presence of numerous pits and tubular gastric glands (Figure D-1-10). A similar region was

Figure D-1-7. Transition zone from esophagus (a) to cardiac/
fundic region of stomach (b) (400X) Bouin's H&E
(c) submucosa; (d) muscularis (smooth muscle);
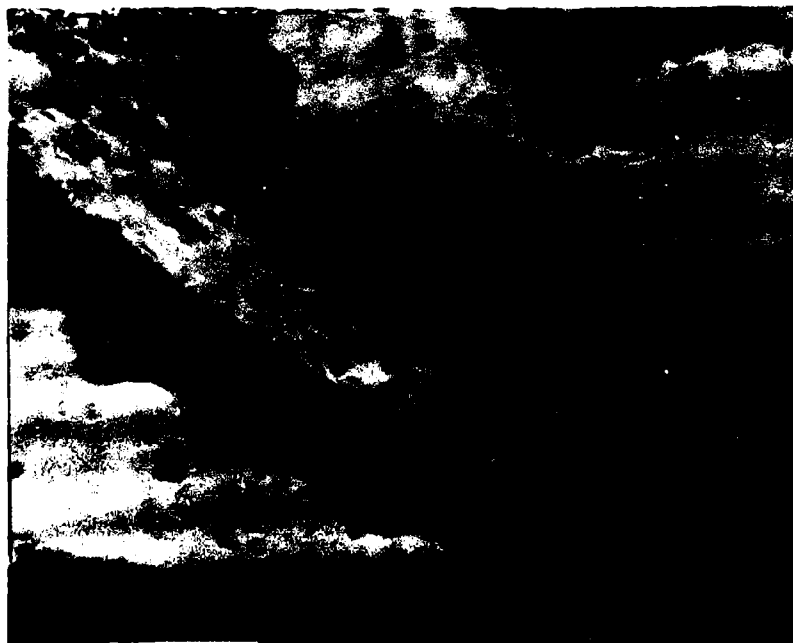(e) serosa.

Figure D-1-8. Cardiac/fundic stomach (400X) Bouin's, H&E.
(a) columnar epithelium; (b) pits.

Figure D-1-9. Anterior fundic/cardiac stomach (400X) Bouin's
H&E. Region of flat mucosa and few gastric pits.

Figure D-1-10. Mid cardiac/fundic stomach (400X) Bouin's H&E.
(a) pits; (b) tubular gastric glands.

noted in the channel catfish and rainbow trout.

The pyloric region consists of sharp-low folds of mucosa. There is a complete absence of gastric glands but a few goblet cells (Figure D-1-11). The pyloric region enters the pyloric intestine via a sphincter (Figure D-1-12). The pyloric caeca of the ascending intestines are numerous. There were no pyloric caeca in the channel catfish (Grizzle and Rogers, 1976).

Ascending and Descending Intestine (Figures D-1-13 and D-1-14)

In the bluegill, the ascending intestine histology differs from the stomach by the absence of a submucosa and muscularis layer in the mucosa. The muscularis and serosa are similar to that of the stomach. The muscularis is very reduced in size in the descending intestine. The mucosa epithelium of the ascending intestine has high peaked folds. There are tall columnar epithelial cells in the mucosa of both ascending and descending intestine. The overall histology of the ascending intestine of the bluegill is similar to the rainbow trout (Anderson and Mitchum, 1974). The mucosal folds of the descending column are more broad and farther apart and there are fewer goblet cells.
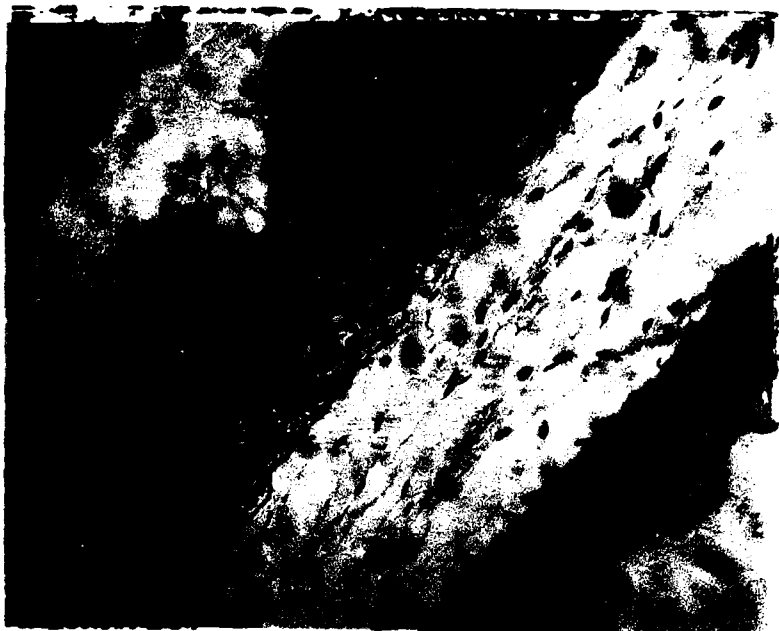
Figure D-1-11.   Pyloric stomach (400X) Bouin's H&E.
(a) goblet cell.

Figure D-1-12.   Sphincter leading to pyloric caeca (100X)
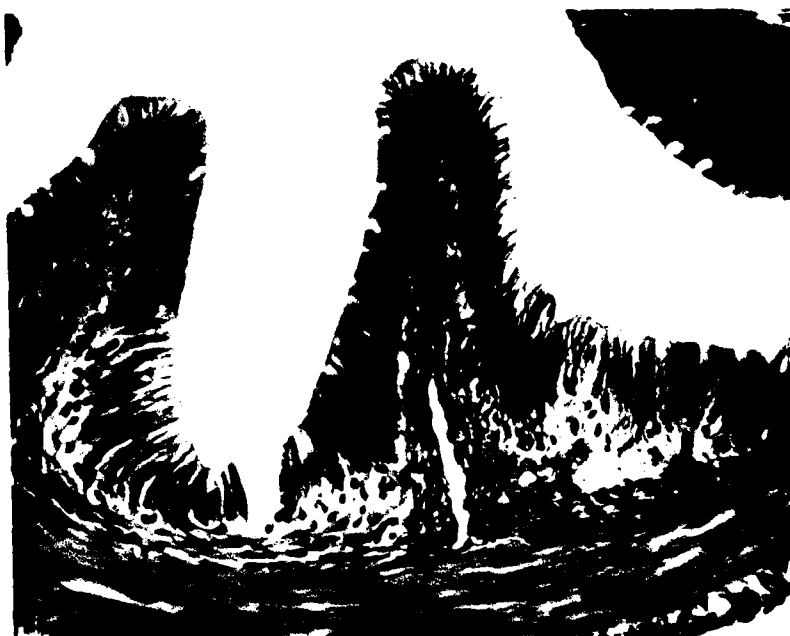Formalin H&E. (a) pyloric caeca; (b) sphinc-
ter muscle.

Figure D-1-13.   Ascending intestine (400X) Bouin's H&E.
(a) mucosa; (b) muscularis; (c) serosa.

Figure D-1-14. Descending intestine (400X) Bouin's H&E.
(a) mucosa; (b) muscularis; (c) serosa.

APPENDIX D-2

PANCREAS HISTOLOGY

The general distribution of pancreatic tissue in the bluegill is similar to the trout (Anderson and Mitchum, 1974). Pancreatic tissue is scattered in the liver around branches of the hepatic vein and along the hepatic portal vein between the liver and spleen (Figure D-2-1). Pancreatic tissue is not found in the spleen as in the channel catfish (Grizzle and Rogers, 1976). Two types of tissue occur. They are the exocrine tissue (acini) and endocrine tissue (islets) as detailed in Figure D-2-2. The endocrine tissue is usually termed islet due to the fact that it is surrounded by acini of exocrine tissue (Anderson and Mitchum, 1974).

The acini of exocrine tissue are usually spherical aggregates of cells. They are composed of pyramidal cells with basally located nuclei (Figure D-2-3) with a centroacinar cell filling the lumen of each group. The secretory substances are carried from these ducts into larger intralobular ducts. The intralobular ducts empty into the phyloric or ascending intestine (Grizzle and Rogers, 1976; Anderson and Mitchum, 1974).

The endocrine islets are cords or masses of cells which are encapsulated by connective tissue (Figure D-2-2). The cords and/or masses of cells are separated by capillaries (Figure D-2-4).
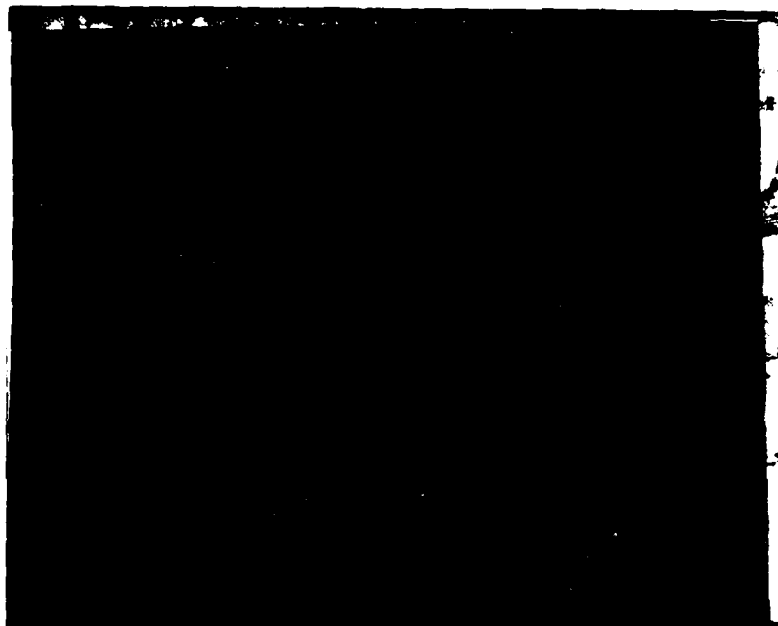
Figure D-2-1. Pancreas (100X) Bouin's H&E. Pancreatic tissue
(a) along the intestine (b) and associated with
the hepatic portal vein (c), exocrine pancreas
(d) and pancreatic islets (endocrine) (e), nerve (f)
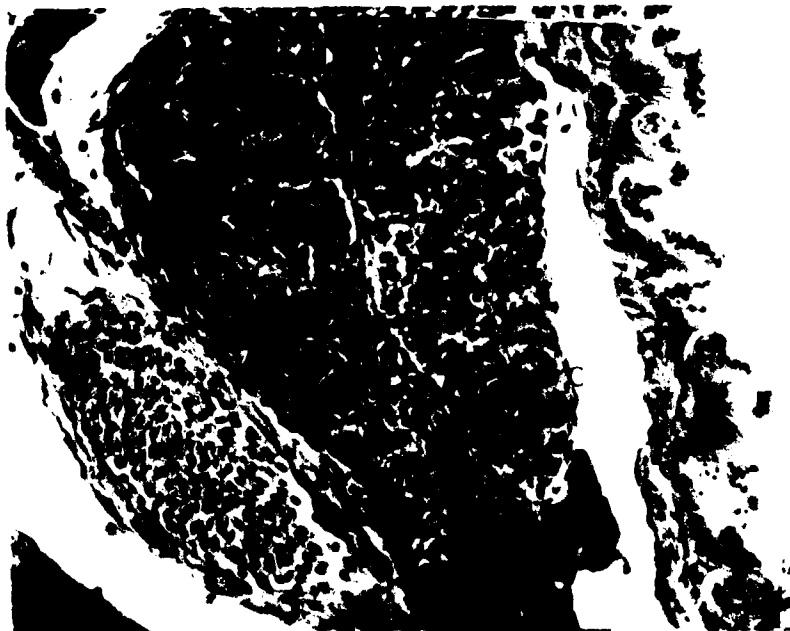
Figure D-2-2. Pancreas (400X) Bouin's H&E. Acini of exocrine
tissue (a), pancreatic islet (b), capsule surround-
ing islet (c), hepatic portal vein (d).

Figure D-2-3. Pancreas (1000X) Bouin's H&E. Acini of exocrine pancreas, spherical aggregates of cells (a) composed of pyramidal cells (b) with basally located nucleus (c) and centroacinar cells filling the lumen.

Figure D-2-4. Pancreas (1000X) Bouin's H&E. Endocrine pancreatic islet tissue. There are cords (a) separated by capillaries (b) and masses of cells (c).

APPENDIX D-3.

INTEGUMENT HISTOLOGY

The integument of the bluegill varies in thickness depending on location. The three layers are the epidermis, dermis and hypodermis (Figure D-3-1). The epidermis may be thin or thick, 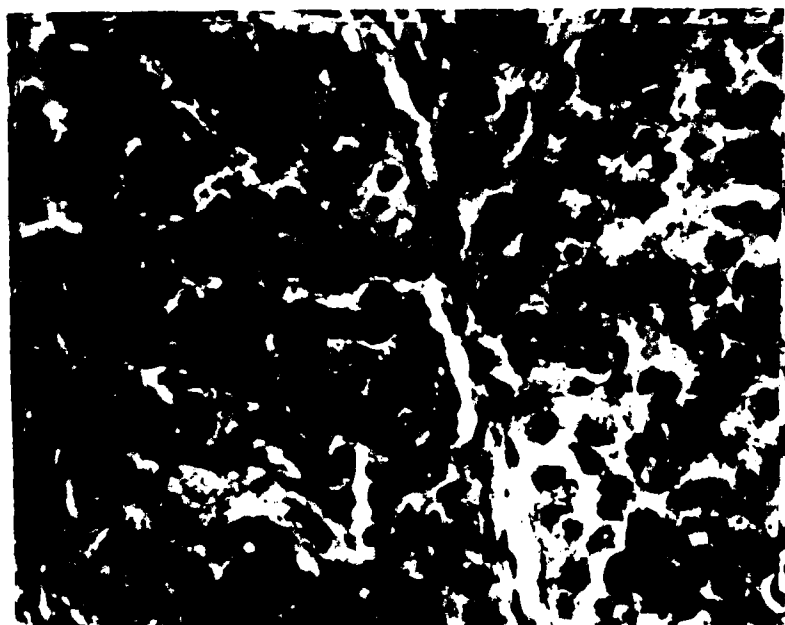slightly keratinized and with numerous mucoid cells (Ashley, 1975). As with trout (Anderson and Mitchum, 1974), the external epidermal layer is composed of squamous epithelial cells with mucous cells interposed (Figure D-3-2). The inner layer of the epidermis is composed of tightly packed germinal cells of irregular shape. An innermost layer of tall columnar cells as present in the channel catfish (Grizzle and Rogers, 1976) and trout (Anderson and Mitchum, 1974) was not present in juvenile bluegill epidermis. The dermis contains scales enclosed in dermal scale pockets. Also present in the dermis are numerous collagenous fibers and melanocytes. The hypodermis is thin at all locations examined. The lateral line is located in the dermis as previously described.
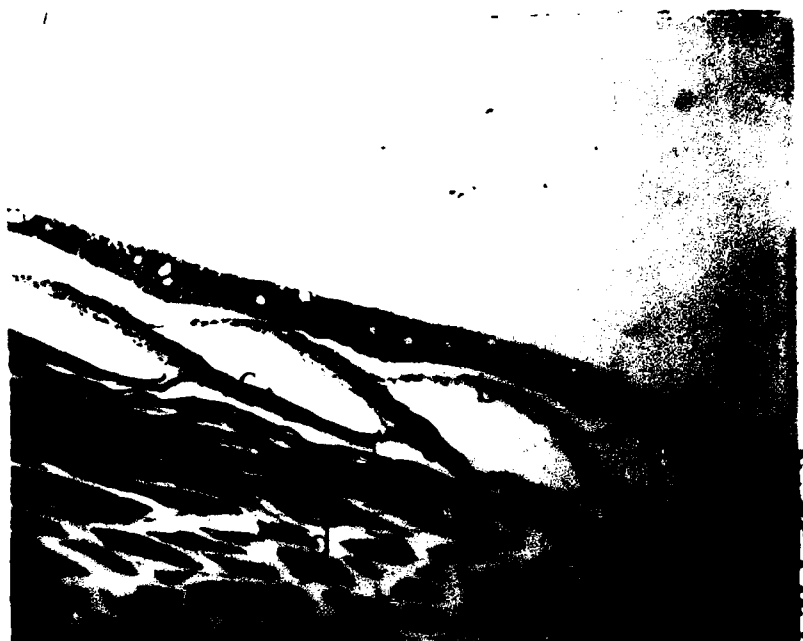
Figure D-3-1. Integument (100X) Formalin, H&E. Epidermis (a), dermis (b), dermal scales (c), striated muscle (d), hypodermis (e).
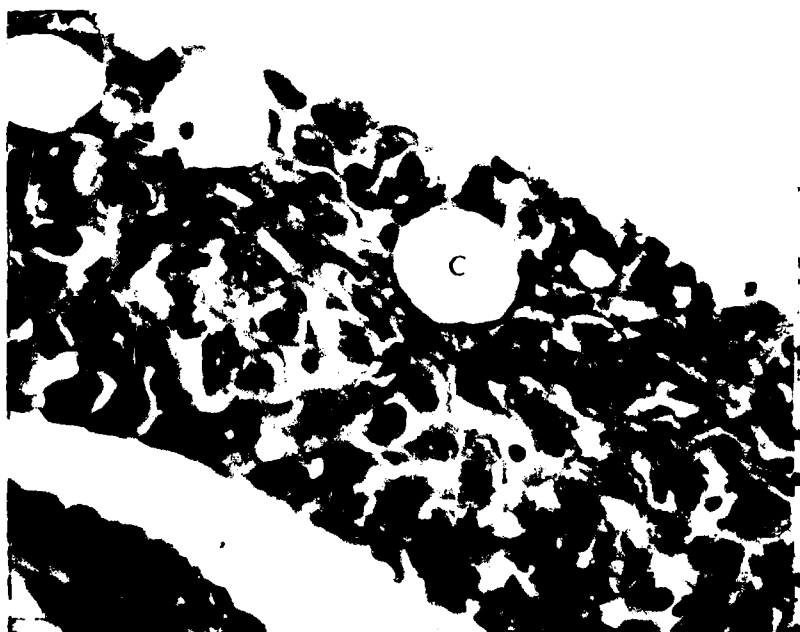
Figure D-3-2. Integument (1000X) Formalin, H&E. Epidermis with stratified squamous epithelium (a) with a basal layer of germinal cells (b). Also present are large mucus secreting cells (c).
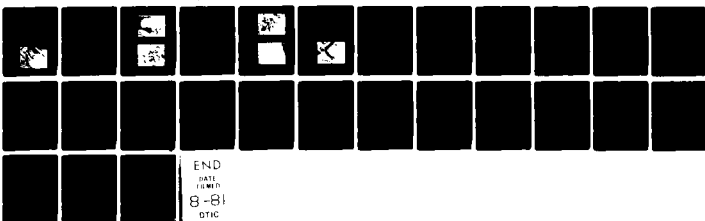
APPENDIX D-4.

HEART HISTOLOGY

The heart of the bluegill is similar to that of the channel catfish (Grizzle and Rogers, 1976) and trout (Anderson and Mitchum, 1974). The heart consists of four chambers (Figure D-4-1). Venus blood from the hepatic vein and paired ducts of Cuvier first enters the sinus venosis and is then pumped into the atrium. The blood then passes to the ventricle. The blood then exits via the bulbus arteriosis. The sinus venosis is thin walled with very little cardiac muscle. The atrium is large composed of cardiac muscle and lined with squamous epithelium as noted in the rainbow trout (Anderson and Mitchum, 1974). The ventricle is made of normal branching cardiac muscle fibers. There are two layers of muscle. The outer layer is compact and the inner layer muscle fibers project into the lumen. The bulbus arteriosis wall is composed of fibrous connective tissue and smooth muscle. There is no cardic muscle in the bulbus arteriosis. This chamber becomes the ventral aorta.

Figure D-4-1. Heart (40X) Bouin's, H&E ventricle (a) sinus venosis (b), atrium (c)

APPENDIX D-5.

GONAD HISTOLOGY

FEMALE:

In the bluegill, the follicle (lamellae) is composed of a single layer of epithelial cells. The tunica albuginea is not completely developed in the juvenile bluegill. The ovary lamellae consist of two layers of oocytes (Figure D-5-1) supported by fibrous connective tissue. The lamellae are shown in longitudinal section in Figure D-5-1. The lumen into which the lamellae project within the poorly developed tunica albuginea leads to the genital pore via an oviduct. The structure is identical to that described for the channel catfish (Grizzle and Rogers, 1976).

MALE:

The testes are composed of seminiferous tubules with spermatogenesis occurring (Figure D-5-2). A fibrous connective tissue (tunica albuginea) surrounds each lobule. Septa separate the tubules in most cases. It is not possible to distinguish an anterior and posterior region in the juvenile bluegill. Spermatozoa are not usually present in the posterior region (Grizzle and Rogers, 1976) of the adult channel catfish.

Figure D-5-1. Ovary (100X) Bouin's H&E. Juvenile ovary showing primary follicles containing oocytes (a) with light staining nuclei and fibrous connective tissue (b).
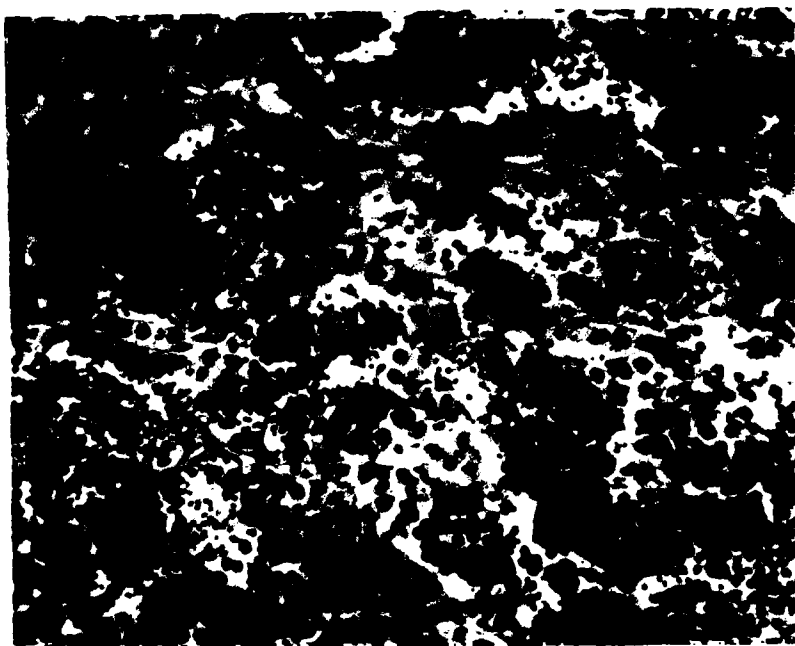
Figure D-5-2. Testis (400X) Bouin's H&E. Seminiferous tubules with spermatozoa (a) filling the lumen of each tubule. Note numerous mitotic figures in the epithelium of the tubules.

APPENDIX D-6.

HEAD KIDNEY

Interrenal hemopoietic and chromaffin tissues are found in the head kidney. Some kidney tubules are present in the juvenile bluegill head kidney. Interrenal and hemopoietic tissues are found in abundance throughout the head kidney (Figure D-6-1). Chromoffin tissue produces adrenalin and noradrenalin (Grizzle and Rogers, 1976). There is a cord-like arrangement (Figure D-6-2) of the large chromaffin tissue cells in the bluegill. The head kidney of the bluegill is very similar to that of the channel catfish (Grizzle and Rogers, 1976). The chromoffin tissue in the trout is more dispersed (Anderson and Mitchum, 1974).
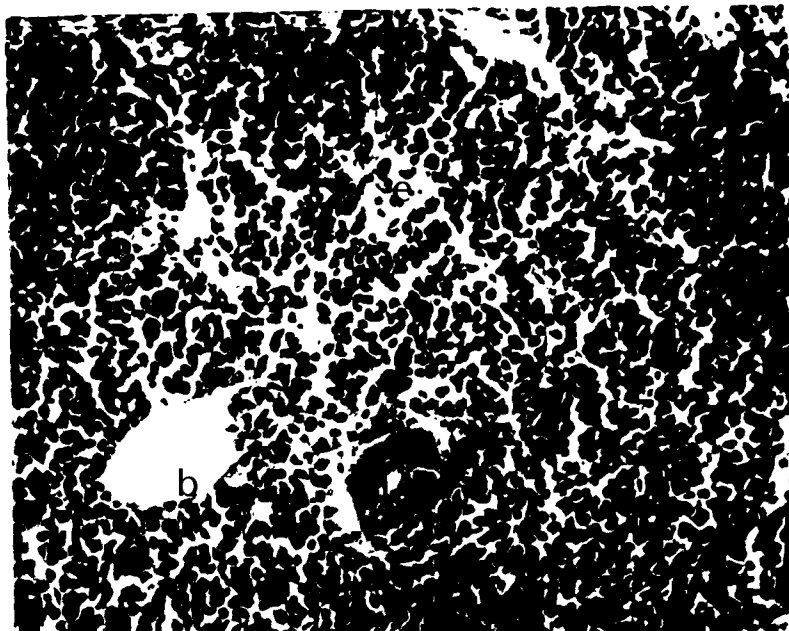
Figure D-6-1. Head kidney (400X) Bouin's H&E. Hemopoietic tissue (a), blood vessel (b), melanin granules (c), kidney tubule (d), interrenal tissue (e).
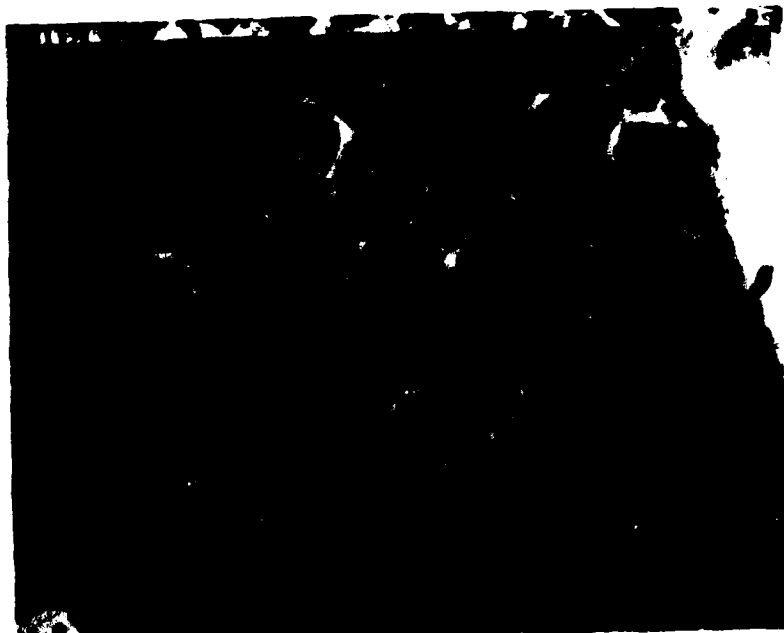
Figure D-6-2. Chromaffin tissue (1000X) Bouin's H&E. Large cells (a) with centrally located nuclei; nerve (b).

APPENDIX D-7.

SPINAL CORD HISTOLOGY

The spinal cord and brain of the bluegill sunfish are similar to that of the channel catfish (Grizzle and Rogers, 1976). The spinal cord has a small central canal. The dorsal horn (Figure D-7-1) is a single mass. There are paired ventral horns separated into an inverted Y similar to that of the rainbow trout (Anderson and Mitchum, 1974). Melanopores are present in the areolar connective tissue surrounding the menix primitiva.

Figure D-7-1. Spinal cord (100X) Formalin, H&E. Central canal (a), dorsal horn of grey matter (b), ventral horn of grey matter (c) and areolar connective tissue (d).

APPENDIX E

2,4-DNT UPTAKE AND LOSS STUDIES

APPENDIX E-1.

CONCENTRATION OF 2,4-DNT IN THE C^{14} RING
LABELED DNT UPTAKE STUDY

Day	dpm*/ml	GAS CHROMATOGRAPHIC ANALYSIS 2,4-DNT mg/l
1	525	2.88
2	540	3.17
3	521	3.01
4	531	3.17
5	528	2.76
6	538	2.89
7	540	2.92
8	552	2.82
9	530	2.74
10	533	2.63
11	524	2.95
12	528	2.64
13	529	3.04
14	533	3.06
Mean	532.3	2.906
SD**	8.06	0.175

*Disintegrations per minute - corrected for machine
efficiency (1.035X)

**Standard deviation

APPENDIX E-2.

QUENCHING AND EFFICIENCY FOR SELECTED
WEIGHTS OF BLUEGILL TISSUES

Tissue	Sample Weight (gm)	Color Ratio	Percent Efficiency	Conversion Factor for Quenching
Whole body	0.10	.794	90.9	1.10
Brain	0.05	.870	100.0	1.00
Kidney	0.10	.715	89.4	1.12
Stomach/ intestine	0.10	.890	81.5	1.23
Gill	0.10	.870	96.0	1.04
Liver	0.10	.794	88.4	1.13
Striated muscle	0.20	.824	93.0	1.08

APPENDIX E-3.

WHOLE BODY UPTAKE AND LOSS OF 2,4-DNT - EXPOSED TO 3.0 mg/l
2,4-DNT FOR TWO WEEKS AND ONE WEEK CLEARING

0.02140 = combined conversion factor

Day	Exposed cpm/0.1gm	Control cpm/0.1gm	cpm/0.1gm Tissue	Bioconcentration Factor	Concentration of 2,4-DNT (μgm/gm tissue)
1	686	561	125	2.575	7.784
1	621	589	32	.684	1.992
2	774	592	182	3.894	11.333
2	893	543	350	7.490	21.795
3	1096	555	541	11.577	33.690
3	1142	560	582	12.454	36.243
4	1481	571	910	19.474	56.669
4	1214	579	635	13.589	39.543
5	1622	524	1098	23.497	68.376
5	1718	570	1148	24.567	71.490
6	1613	557	1056	22.598	65.761
6	1631	527	1104	23.625	68.750
7	1587	556	1031	22.063	64.204
7	1960	568	1392	29.788	86.685
8	1823	532	1231	27.627	80.395
8	1768	560	1208	25.851	77.227
9	1654	609	1045	22.363	65.076
9	1755	514	1241	26.557	77.282

APPENDIX E-3. (cont.)

Day	Exposed cpm/0.1gm	Control cpm/0.1gm	cpm/0.1gm Tissue	Bioconcentration Factor	Concentration of 2,4-DNT (μgm/gm tissue)
10	1773	584	1189	25.444	74.044
10	1828	552	1276	27.306	79.461
11	1657	523	1134	24.267	70.618
11	1693	607	1086	23.240	67.629
12	1757	623	1134	24.267	70.618
12	2164	543	1621	34.689	100.946
13	1571	554	1071	21.763	63.332
13	1555	603	952	20.372	59.284
14	1577	548	1029	22.020	64.079
14	1691	586	1105	23.647	68.812
1	1444	609	835	17.869	51.998
1	1676	644	1012	21.656	63.021
2	1102	655	447	9.565	27.836
2	963	669	294	6.292	18.309
3	723	583	140	2.996	8.718
3	770	664	105	2.247	6.538
4	658	599	59	--	--
4	543	611	--	--	--
5	541	599	--	--	--
5	566	598	--	--	--
6	561	570	--	--	--
6	478	619	--	--	--
7	498	634	--	--	--
7	543	620	--	--	--

APPENDIX E-4.

BRAIN UPTAKE AND LOSS OF 2,4-DNT - EXPOSED TO 3.0 mg/l 2,4-DNT FOR TWO WEEKS AND ONE WEEK CLEARING

0.03891 = combined conversion factor

Day	Exposed cpm/0.05gm	Control cpm/0.05gm	cpm/0.05gm Tissue	Bioconcentration Factor	Concentration of 2,4-DNT (μgm/gm tissue)
1	699	582	117	4.552	13.248
2	673	568	105	4.085	11.889
3	919	554	365	14.202	41.328
4	1992	566	1425	55.486	161.463
5	1312	549	763	29.688	86.39
6	2111	558	1553	60.427	175.843
7	3276	650	2626	102.177	297.336
8	3216	578	2638	102.644	298.695
9	3288	537	2715	105.640	307.414
10	3263	595	2668	103.812	302.093
11	3179	554	2625	102.138	297.223
12	3074	552	2522	98.131	285.563
13	3028	533	2495	97.804	282.504
14	3370	507	2863	111.399	324.172
1	2509	518	1991	77.470	225.437
2	1741	541	1200	46.692	135.873
3	1364	597	767	29.843	86.846
4	661	596	65	2.529	7.359
5	619	605	-	-	-
6	552	617	-	-	-
7	529	649	-	-	-

APPENDIX E-5.

KIDNEY UPTAKE AND LOSS OF 2,4-DNT - EXPOSED TO 3.0 mg/l 2,4-DNT FOR TWO WEEKS AND ONE WEEK CLEARING

0.02179 = combined conversion factor

Day	Exposed cpm/0.1gm	Control cpm/0.1gm	cpm/0.1gm Tissue	Bioconcentration Factor	Concentration of 2,4-DNT (μgm/gm tissue)
1	714	520	194	4.227	12.301
2	865	555	310	6.755	19.655
3	1147	580	567	12.354	35.951
4	1475	530	945	20.591	59.919
5	1902	526	1376	29.982	87.247
6	2430	574	1856	40.440	117.681
7	2966	593	2373	51.705	150.460
8	2699	582	2117	46.127	134.230
9	2848	567	2281	49.701	144.629
10	2893	541	2352	51.247	149.130
11	2588	587	2001	43.599	126.875
12	2864	557	2307	50.267	146.278
13	2496	561	1935	42.161	122.690
14	2954	604	2350	51.204	149.004
1	2714	564	2150	46.846	136.323
2	1626	602	1024	22.312	64.927
3	1361	569	732	17.256	50.218
4	724	655	69	1.503	4.375
5	761	577	194	4.009	11.666
6	581	629	-	-	-
7	522	646	-	-	-

APPENDIX E-6.

STOMACH/INTESTINE UPTAKE AND LOSS OF 2,4-DNT - EXPOSED TO 3.0 mg/1 2,4-DNT FOR TWO WEEKS AND ONE WEEK CLEARING

0.02393 = combined conversion factor

Day	Exposed cpm/0.1gm	Control cpm/0.1gm	cpm/0.1gm Tissue	Bioconcentration Factor	Concentration of 2,4-DNT (μgm/gm tissue)
1	784	599	185	4.427	12.883
2	807	662	145	3.469	10.097
3	1294	623	671	16.057	46.726
4	1998	537	1461	34.962	101.739
5	1601	564	1037	24.815	72.213
6	1743	605	1138	27.232	79.246
7	1957	542	1415	33.861	98.535
8	1873	553	1320	31.588	91.919
9	1792	558	1234	29.529	85.931
10	1807	573	1234	29.529	85.931
11	1739	576	1163	27.831	80.987
12	1784	591	1193	28.548	83.076
13	1602	599	1003	24.002	69.845
14	1791	560	1231	29.458	85.722
1	1668	578	1090	26.084	75.904
2	1646	543	1103	26.395	76.809
3	1054	581	473	11.319	32.938
4	730	609	121	2.896	8.425
5	560	643	—	—	—
6	597	636	—	—	—
7	506	604	—	—	—

APPENDIX E-7.

GILL UPTAKE AND LOSS OF 2,4-DNT - EXPOSED TO 3.0 mg/1 2,4-DNT FOR TWO WEEKS AND ONE WEEK CLEARING

0.02023 = combined conversion factor

Day	Exposed cpm/0.1gm	Control cpm/0.1gm	cpm/0.1gm Tissue	Bioconcentration Factor	Concentration of 2,4-DNT (μgm/gm tissue)
1	688	559	129	2.509	7.594
2	793	541	252	5.098	14.835
3	1116	595	521	10.539	30.670
4	1533	565	969	19.582	56.985
5	1581	599	982	19.865	57.809
6	1593	557	1036	20.958	60.988
7	1715	560	1155	23.366	67.994
8	1610	531	1079	21.828	63.519
9	1717	567	1150	23.264	67.699
10	1789	551	1233	25.045	72.880
11	1748	584	1164	23.547	68.523
12	1723	609	1114	22.536	65.580
13	2299	555	1744	35.281	102.668
14	1823	604	1219	24.660	71.762
1	1659	618	1039	21.018	61.165
2	1448	619	829	16.770	48.800
3	791	613	178	3.601	10.478
4	552	552	–	–	–
5	546	573	–	–	–
6	522	644	–	–	–
7	553	649	–	–	–

APPENDIX E-8.

LIVER-PANCREAS UPTAKE AND LOSS OF 2,4-DNT – EXPOSED TO 3.0 mg/l
2,4-DNT FOR TWO WEEKS AND ONE WEEK CLEARING

0.02198 = combined conversion factor

Day	Exposed cpm/0.1gm	Control cpm/0.1gm	cpm/0.1gm Tissue	Bioconcentration Factor	Concentration of 2,4-DNT (μgm/gm tissue)
1	644	543	101	2.220	6.461
2	871	605	266	5.847	17.014
3	759	588	171	3.759	10.938
4	1214	510	704	15.474	45.029
5	1213	551	662	14.551	42.343
6	1776	542	1234	27.123	78.929
7	1974	607	1367	30.047	87.436
8	2021	579	1442	31.695	92.233
9	1869	514	1355	29.783	86.668
10	1998	558	1440	31.651	92.105
11	1831	498	1333	29.299	85.261
12	1671	596	1075	23.629	68.759
13	1926	531	1395	30.662	89.227
14	1885	555	1330	29.233	85.069
1	1401	561	840	18.463	53.728
2	1113	618	495	10.880	31.661
3	694	529	165	3.620	10.554
4	560	588	–	–	–
5	566	541	–	–	–
6	571	619	–	–	–
7	543	648	–	–	–

APPENDIX E-9

STRIATED MUSCLE UPTAKE AND LOSS OF 2,4-DNT – EXPOSED TO 3.0 mg/1
2,4-DNT FOR TWO WEEKS AND ONE WEEK CLEARING

0.0150 = combined conversion factor

Day	Exposed cpm/0.2gm	Control cpm/0.2gm	cpm/0.2gm Tissue	Bioconcentration Factor	Concentration of 2,4-DNT (μgm/gm tissue)
1	663	546	117	1.228	3.574
2	768	556	212	2.226	6.477
3	994	541	453	4.756	13.841
4	1352	568	784	8.232	23.955
5	1472	546	926	9.723	28.293
6	1587	586	1001	10.511	30.585
7	1740	532	1208	12.684	36.914
8	1493	579	914	9.597	27.927
9	1699	532	1167	12.253	35.657
10	1592	597	997	10.447	30.402
11	1647	537	1110	11.655	33.916
12	1497	564	933	9.796	28.507
13	1244	529	715	7.508	21.847
14	1692	570	1122	11.781	34.282
1	1611	631	980	10.290	29.944
2	1156	602	554	5.817	16.927
3	658	614	44	0.467	1.341
4	609	578	31	0.325	0.947
5	566	581	–	–	–
6	554	566	–	–	–
7	539	655	–	–	–

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